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(54) Title: NUCLEIC ACID MOLECULE ENCODING A MISMATCH ENDONUCLEASE AND METHODS OF USE THEREOF

(57) Abstract: Nucleic acid molecules encoding a mismatch endonuclease and its method of use for the detection of mutations in targeted polynucleotide sequences are provided, which facilitate the localization and identification of mutations, mismatches and genetic polymorphisms.

**NUCLEIC ACID MOLECULE ENCODING A MISMATCH ENDONUCLEASE
AND METHODS OF USE THEREOF**

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Pursuant to 35 U.S.C. §202(c) it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the National Institutes of Health, Grant Number, NIH CA71426.

FIELD OF THE INVENTION

This invention relates to materials and methods for the detection of mutations in targeted nucleic acids. More specifically, the invention provides nucleic acid molecules encoding a mismatch specific nuclease and methods of use of the enzyme that facilitate the genetic screening of hereditary diseases and cancer. The method is also useful for the detection of genetic polymorphisms.

10

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by numerals in parenthesis in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference in the present specification.

The sequence of nucleotides within a gene can be mutationally altered or "mismatched" in any of several ways, the most frequent of which being base-pair substitutions, frame-shift mutations and deletions or insertions. These mutations can be induced by environmental factors, such as radiation and mutagenic chemicals; errors are also occasionally committed by DNA polymerases during replication. Many human disease

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5 states arise because fidelity of DNA replication is not maintained. Cystic fibrosis, sickle cell anemia and some cancers are caused by single base changes in the DNA resulting in the synthesis of aberrant or non-functional proteins.

10 The high growth rate of plants and the abundance of DNA intercalators in plants suggests an enhanced propensity for mismatch and frameshift lesions. Plants and fungi are known to possess an abundance of single-stranded specific nucleases that attack both DNA
15 and RNA (9-14). Some of these, like the Nuclease α of *Ustilago maydis*, are suggested to take part in gene conversion during DNA recombination (15,16). Of these nucleases, S1 nuclease from *Aspergillus oryzae* (17), and P1 nuclease from *Penicillium citrinum* (18), and Mung
20 Bean Nuclease from the sprouts of *Vigna radiata* (19-22) are the best characterized. S1, P1 and the Mung Bean Nuclease are Zn proteins active mainly near pH 5.0 while Nuclease α is active at pH 8.0. The single strandedness property of DNA lesions appears to have been used by a
25 plant enzyme, SP nuclease, for bulky adduct repair. The nuclease SP, purified from spinach, is a singlestranded DNase, an RNase, and able to incise DNA at TC₆₋₄ dimers and cisplatin lesions, all at neutral pH (23,24).

In *Escherichia coli*, lesions of
30 base-substitution and unpaired DNA loops are repaired by a methylation-directed long patch repair system. The proteins in this multienzyme system include MutH, MutL and MutS (1, 2). This system is efficient, but the C/C lesion and DNA loops larger than 4 nucleotides are not
35 repaired. The MutS and MutL proteins are conserved from bacteria to humans, and appear to be able to perform similar repair roles in higher organisms. For some of the lesions not well repaired by the MutS/MutL system,

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5 and for gene conversion where short-patch repair systems may be more desirable, other mismatch repair systems with novel capabilities are needed.

Currently, the most direct method for mutational analysis is DNA sequencing, however it is
10 also the most labor intensive and expensive. It is usually not practical to sequence all potentially relevant regions of every experimental sample. Instead some type of preliminary screening method is commonly used to identify and target for sequencing only those
15 samples that contain mutations. Single stranded conformational polymorphism (SSCP) is a widely used screening method based on mobility differences between single-stranded wild type and mutant sequences on native polyacrylamide gels. Other methods are based on
20 mobility differences in wild type/mutant heteroduplexes (compared to control homoduplexes) on native gels (heteroduplex analysis) or denaturing gels (denaturing gradient gel electrophoresis). While sample preparation is relatively easy in these assays, very exacting
25 conditions for electrophoresis are required to generate the often subtle mobility differences that form the basis for identifying the targets that contain mutations. Another critical parameter is the size of the target region being screened. In general, SSCP is
30 used to screen target regions no longer than about 200-300 bases. The reliability of SSCP for detecting single-base mutations is somewhat uncertain but is probably in the 70-90% range for targets less than 200 bases. As the size of the target region increases, the
35 detection rate declines, for example in one study from 87% for 183 bp targets to 57% for targets 307 bp in length (35). The ability to screen longer regions in a single step would enhance the utility of any mutation

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5 screening method.

Another type of screening technique currently in use is based on cleavage of unpaired bases in heteroduplexes formed between wild type probes hybridized to experimental targets containing point mutations. The cleavage products are also analyzed by gel electrophoresis, as subfragments generated by cleavage of the probe at a mismatch generally differ significantly in size from full length, uncleaved probe and are easily detected with a standard gel system. Mismatch cleavage has been effected either chemically (osmium tetroxide, hydroxylamine) or with a less toxic, enzymatic alternative, using RNase A. The RNase A cleavage assay has also been used, although much less frequently, to screen for mutations in endogenous mRNA targets or for detecting mutations in DNA targets amplified by PCR. A mutation detection rate of over 50% was reported for the original RNase screening method (36).

A newer method to detect mutations in DNA relies on DNA ligase which covalently joins two adjacent oligonucleotides which are hybridized on a complementary target nucleic acid. The mismatch must occur at the site of ligation. As with other methods that rely on oligonucleotides, salt concentration and temperature at hybridization are crucial. Another consideration is the amount of enzyme added relative to the DNA concentration.

The methods mentioned above cannot reliably detect a base change in a nucleic acid which is contaminated with more than 80% of a background nucleic acid, such as normal or wild type sequences. Contamination problems are significant in cancer detection wherein a malignant cell, in circulation for

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5 example, is present in extremely low amounts. The methods now in use lack adequate sensitivity to be practically applied in the clinical setting.

A method for the detection of gene mutations with mismatch repair enzymes has been described by Lu-
10 Chang and Hsu. See WO 93/20233. The product of the MutY gene which recognizes mispaired A/G residues is employed in conjunction with another enzyme described in the reference as an "all type enzyme" which can nick at all base pair mismatches. The enzyme does not detect
15 insertions and deletions. Also, the all type enzyme recognizes different mismatches with differing efficiencies and its activity can be adversely affected by flanking DNA sequences. This method therefore relies on a cocktail of mismatch repair enzymes and/or
20 combinations of DNA glycosylases to detect the variety of mutations that can occur in a given DNA molecule.

SUMMARY OF THE INVENTION

The present invention provides materials and
25 methods for the detection of mutations or mismatches in a targeted polynucleotide strand. Nucleic acid molecules encoding a mismatch endonuclease and methods of use thereof are disclosed. Detection is achieved using an endonuclease encoded by the nucleic acid
30 molecules of the invention in combination with a gel assay system that facilitates the screening and identification of altered base pairing in a targeted nucleic acid strand. The availability of the nucleic acid having the sequence of SEQ ID NO:1 facilitates the
35 preparation of large amounts of purified CEL I enzyme for use in such an assay.

In a preferred embodiment of the invention, an isolated nucleic acid molecule having the sequence of

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5 SEQ ID NO:1 encoding an endonuclease protein from celery about 43 kDa and 309 amino acids in length is provided. The endonuclease protein comprises a plurality of α helical domains and a flexible carboxy terminal region. The nucleic acid may be DNA or cDNA.

10 DNA molecules for isolating genomic clones of the invention are also provided. Such sequences facilitate the identification and cloning of a CEL I gene comprising introns and exons, the exons encoding the CEL 1 protein and specifically hybridizing with the
15 nucleic acid of SEQ ID NO:1. Isolated RNA molecules transcribed from the nucleic acid of SEQ ID NO: 1 are also within the scope of the present invention.

In another aspect of the invention, a polynucleotide which comprises a) a sequence encoding a
20 protein or polypeptide having SEQ ID NO: 2; b) a sequence encoding the complementary sequence of a); b) a sequence of nucleotides shown in Figure 2; and c) a fragment of any of the sequences in a), or b) is disclosed.

25 In a preferred embodiment of the invention, an oligonucleotide between about 10 and about 200 nucleotides in length, which specifically hybridizes with SEQ ID NO:1 is provided.

In yet another aspect, an antibody
30 immunologically specific for the isolated CEL I protein is provided. The antibody may be monoclonal or polyclonal.

Plasmids and vectors comprising SEQ ID NO: 1 are also within the scope of the present invention. In
35 one embodiment, the vector may be a retroviral vector.

In a preferred embodiment of the invention, the plasmids or vectors described above may be introduced into host cells. Host cells suitable for

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5 this purpose include, without limitation, bacterial cells, plant cells, insect cells, procaryotic cells, fungal and mammalian cells.

Transgenic animals comprising SEQ ID NO: 1 are included in the present invention and have utility for
10 assessing CEL I activities in a mammalian milieu.

Methods employing the nucleic acids of the invention are also provided. In one embodiment, a method for screening test compounds for CEL I modulating activity are provided. A host cell expressing a CEL I
15 encoding nucleic acid is provided. The host cell is then contacted with a compound suspected of modulating CEL I activity and CEL I modulating activity is assessed by an alteration in the endonuclease activity of CEL I.

In a particularly preferred embodiment of the invention, a method for determining a mutation in a
20 target sequence of single stranded polynucleotide with reference to a non-mutated sequence of a polynucleotide that is hybridizable with the polynucleotide including the target sequence is disclosed. The sequences are
25 amplified, labeled with a detectable marker, hybridized to one another, exposed to a plant endonuclease encoded by a nucleic acid molecule having greater than >60% identity to a nucleic acid having the sequence of SEQ ID NO: 1, and analyzed for the presence of the mutation.

30 In an alternative embodiment, the endonuclease is CEL I and is encoded by SEQ ID NO: 1. The availability of a nucleic acid having a sequence of SEQ ID NO: 1 facilitates the production of large quantities of the CEL I endonuclease for use in the method above.

35 Exemplary endonucleases having greater than 60% sequence identity to CEL I are encoded by ZEN1 from *Zinnia*, BFN1 from *Arabidopsis* and DSA6 from daylily.

Mismatch-specific nucleases corresponding to

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5 CEL I have been detected in more than 14 plant species.
It is therefore anticipated that many additional plants
contain nuclease genes that produce a protein with a
high percentage of identity to SEQ ID NO:2. This use of
these ortholog nuclease sequences to produce CEL I-like
10 activity is contemplated with regard to the present
invention. The encoded CEL I nuclease and its orthologs
possess the following activities: i) detection of all
mismatches between said hybridized sequences; ii)
recognition of sequence differences in polynucleotide
15 strands between about 100bp and about 3kb in length; and
iii) recognition of said mutation in a target
polynucleotide sequence without substantial adverse
effect caused by flanking polynucleotide sequences.

DNA molecules and cDNA molecules may be
20 assessed in the method described above. The method may
be used to advantage in the screening assays for
identifying alterations in DNA associated with genetic
diseases and predisposition to cancer.

In yet another embodiment of the invention, an
25 isozyme of CEL I having endonuclease activity is
provided. The CEL I isozyme has a molecular weight of
39 kd and is isolated from celery.

In order to more clearly set forth the
parameters of the present invention, the following
30 definitions are used:

The term "endonuclease" refers to an enzyme
that can cleave DNA internally.

The term "base pair mismatch" indicates a base
pair combination that generally does not form in nucleic
35 acids according to Watson and Crick base pairing rules.
For example, when dealing with the bases commonly found
in DNA, namely adenine, guanine, cytosine and thymidine,
base pair mismatches are those base combinations other

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5 than the A-T and G-C pairs normally found in DNA. As described herein, a mismatch may be indicated, for example as C/C meaning that a cytosine residue is found opposite another cytosine, as opposed to the proper pairing partner, guanine.

10 The phrase "DNA insertion or deletion" refers to the presence or absence of "matched" bases between two strands of DNA such that complementarity is not maintained over the region of inserted or deleted bases.

15 The term "complementary" refers to two DNA strands that exhibit substantial normal base pairing characteristics. Complementary DNA may contain one or more mismatches, however.

20 The phrase "flanking nucleic acid sequences" refers to those contiguous nucleic acid sequences that are 5' and 3' to the endonuclease cleavage site.

The term "multiplex analysis" refers to the simultaneous assay of pooled DNA samples according to the above described methods.

25 C>T indicates the substitution of a thymidine residue for a cytosine residue giving rise to a mismatch. Inappropriate substitution of any base for another giving rise to a mismatch or a polymorphism may be indicated this way.

30 N, N, N', N'-tetramethyl-6-carboxyrhodamine (TAMRA) is a fluorescent dye used to label DNA molecular weight standards which are in turn utilized as an internal standard for DNA analyzed by automated DNA sequencing.

35 Primers may be labeled fluorescently with 6-carboxyfluorescein (6-FAM). Alternatively primers may be labeled with 4, 7, 2', 7'-Tetrachloro-6-carboxyfluorescein (TET). Other alternative DNA

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5 labeling methods are known in the art and are contemplated to be within the scope of the invention.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear
10 or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the
15 invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For
20 example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

25 When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural
30 state (i.e., in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during
35 its production.

"Natural allelic variants", "mutants" and "derivatives" of particular sequences of nucleic acids refer to nucleic acid sequences that are closely related

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5 to a particular sequence but which may possess, either naturally or by design, changes in sequence or structure. By closely related, it is meant that at least about 60%, but often, more than 85%, of the nucleotides of the sequence match over the defined
10 length of the nucleic acid sequence referred to using a specific SEQ ID NO. Changes or differences in nucleotide sequence between closely related nucleic acid sequences may represent nucleotide changes in the sequence that arise during the course of normal
15 replication or duplication in nature of the particular nucleic acid sequence. Other changes may be specifically designed and introduced into the sequence for specific purposes, such as to change an amino acid codon or sequence in a regulatory region of the nucleic
20 acid. Such specific changes may be made *in vitro* using a variety of mutagenesis techniques or produced in a host organism placed under particular selection conditions that induce or select for the changes. Such sequence variants generated specifically may be referred
25 to as "mutants" or "derivatives" of the original sequence.

The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the
30 University of Wisconsin GCG software program and are further discussed below.

The present invention also includes active portions, fragments, derivatives and functional or non-functional mimetics of CEL I polypeptides or
35 proteins of the invention. An "active portion" of CEL I polypeptide means a peptide that is less than the full length CEL I polypeptide, but which retains measurable biological activity.

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5 A "fragment" or "portion" of the CEL I
polypeptide means a stretch of amino acid residues of at
least about five to seven contiguous amino acids, often
at least about seven to nine contiguous amino acids,
typically at least about nine to thirteen contiguous
10 amino acids and, most preferably, at least about twenty
to thirty or more contiguous amino acids. A
"derivative" of the CEL I polypeptide or a fragment
thereof means a polypeptide modified by varying the
amino acid sequence of the protein, e.g. by manipulation
15 of the nucleic acid encoding the protein or by altering
the protein itself. Such derivatives of the natural
amino acid sequence may involve insertion, addition,
deletion or substitution of one or more amino acids, and
may or may not alter the essential activity of the
20 original CEL I polypeptide.

Different "variants" of the CEL I polypeptide
exist in nature. These variants may be alleles
characterized by differences in the nucleotide sequences
of the gene coding for the protein, or may involve
25 different RNA processing or post-translational
modifications. The skilled person can produce variants
having single or multiple amino acid substitutions,
deletions, additions or replacements. These variants may
include inter alia: (a) variants in which one or more
30 amino acids residues are substituted with conservative
or non-conservative amino acids, (b) variants in which
one or more amino acids are added to the CEL I
polypeptide, (c) variants in which one or more amino
acids include a substituent group, and (d) variants in
35 which the CEL I polypeptide is fused with another
peptide or polypeptide such as a fusion partner, a
protein tag or other chemical moiety, that may confer
useful properties to the CEL I polypeptide, such as, for

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5 example, an epitope for an antibody, a polyhistidine
sequence, a biotin moiety and the like. Other CEL I
polypeptides of the invention include variants in which
amino acid residues from one species are substituted for
the corresponding residue in another species, either at
10 the conserved or non-conserved positions. In another
embodiment, amino acid residues at non-conserved
positions are substituted with conservative or
non-conservative residues. The techniques for obtaining
these variants, including genetic (suppressions,
15 deletions, mutations, etc.), chemical, and enzymatic
techniques are known to the person having ordinary skill
in the art.

To the extent such allelic variations,
analogues, fragments, derivatives, mutants, and
20 modifications, including alternative nucleic acid
processing forms and alternative post-translational
modification forms result in derivatives of the CEL I
polypeptide that retain any of the biological properties
of the CEL I polypeptide, they are included within the
25 scope of this invention.

The term "orthologs" as used herein refers to
nucleases encoded by nucleic acid sequences whose
polypeptide product has greater than 60% identity to the
CEL I encoding sequence and whose gene products have
30 similar three dimensional structure and biochemical
activities of CEL I. The use of nucleases encoded by
such orthologs in the methods of the invention is
contemplated herein. Exemplary orthologs include,
without limitation, ZEN1, BFN1 and DSA6.

35 The term "functional" as used herein implies that
the nucleic or amino acid sequence is functional for the
recited assay or purpose.

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5 The phrase "consisting essentially of" when
referring to a particular nucleotide or amino acid
means a sequence having the properties of a given SEQ ID
No:. For example, when used in reference to an amino
acid sequence, the phrase includes the sequence per se
10 and molecular modifications that would not affect the
basic and novel characteristics of the sequence.

A "replicon" is any genetic element, for example, a
plasmid, cosmid, bacmid, phage or virus, that is capable
of replication largely under its own control. A replicon
15 may be either RNA or DNA and may be single or double
stranded.

A "vector" is a replicon, such as a plasmid,
cosmid, bacmid, phage or virus, to which another genetic
sequence or element (either DNA or RNA) may be attached
20 so as to bring about the replication of the attached
sequence or element.

An "expression operon" refers to a nucleic acid
segment that may possess transcriptional and
translational control sequences, such as promoters,
25 enhancers, translational start signals (e.g., ATG or AUG
codons), polyadenylation signals, terminators, and the
like, and which facilitate the expression of a
polypeptide coding sequence in a host cell or organism.

The term "oligonucleotide," as used herein refers
30 to primers and probes of the present invention, and is
defined as a nucleic acid molecule comprised of two or
more ribo- or deoxyribonucleotides, preferably more than
three. The exact size of the oligonucleotide will
depend on various factors and on the particular
35 application and use of the oligonucleotide.

The term "probe" as used herein refers to an
oligonucleotide, polynucleotide or nucleic acid, either
RNA or DNA, whether occurring naturally as in a purified

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5 restriction enzyme digest or produced synthetically,
which is capable of annealing with or specifically
hybridizing to a nucleic acid with sequences
complementary to the probe. A probe may be either
single-stranded or double-stranded. The exact length of
10 the probe will depend upon many factors, including
temperature, source of probe and use of the method. For
example, for diagnostic applications, depending on the
complexity of the target sequence, the oligonucleotide
probe typically contains 15-25 or more nucleotides,
15 although it may contain fewer nucleotides. The probes
herein are selected to be "substantially" complementary
to different strands of a particular target nucleic acid
sequence. This means that the probes must be
sufficiently complementary so as to be able to
20 "specifically hybridize" or anneal with their respective
target strands under a set of pre-determined conditions.
Therefore, the probe sequence need not reflect the exact
complementary sequence of the target. For example, a
non-complementary nucleotide fragment may be attached to
25 the 5' or 3' end of the probe, with the remainder of the
probe sequence being complementary to the target strand.
Alternatively, non-complementary bases or longer
sequences can be interspersed into the probe, provided
that the probe sequence has sufficient complementarity
30 with the sequence of the target nucleic acid to anneal
therewith specifically.

The term "specifically hybridize" refers to the
association between two single-stranded nucleic acid
molecules of sufficiently complementary sequence to
35 permit such hybridization under pre-determined
conditions generally used in the art (sometimes termed
"substantially complementary"). In particular, the term
refers to hybridization of an oligonucleotide with a

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5 substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

10 The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed
15 in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme,
20 suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length
25 depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to
30 prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or
35 similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise

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5 complementary primer. Alternatively, non-complementary
bases may be interspersed within the oligonucleotide
primer sequence, provided that the primer sequence has
sufficient complementarity with the sequence of the
desired template strand to functionally provide a
10 template-primer complex for the synthesis of the
extension product.

The term "isolated protein" or "isolated and
purified protein" is sometimes used herein. This term
refers primarily to a protein produced by expression of
15 an isolated nucleic acid molecule of the invention.
Alternatively, this term may refer to a protein that has
been sufficiently separated from other proteins with
which it would naturally be associated, so as to exist
in "substantially pure" form. "Isolated" is not meant to
20 exclude artificial or synthetic mixtures with other
compounds or materials, or the presence of impurities
that do not interfere with the fundamental activity, and
that may be present, for example, due to incomplete
purification, addition of stabilizers, or compounding
25 into, for example, immunogenic preparations or
pharmaceutically acceptable preparations.

The term "substantially pure" refers to a
preparation comprising at least 50-60% by weight of a
given material (e.g., nucleic acid, oligonucleotide,
30 protein, etc.). More preferably, the preparation
comprises at least 75% by weight, and most preferably
90-95% by weight of the given compound. Purity is
measured by methods appropriate for the given compound
(e.g. chromatographic methods, agarose or polyacrylamide
35 gel electrophoresis, HPLC analysis, and the like).

"Mature protein" or "mature polypeptide" shall mean
a polypeptide possessing the sequence of the polypeptide
after any processing events that normally occur to the

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5 polypeptide during the course of its genesis, such as
protoolytic processing from a polyprotein precursor. In
designating the sequence or boundaries of a mature
protein, the first amino acid of the mature protein
sequence is designated as amino acid residue 1.

10 The term "tag," "tag sequence" or "protein tag"
refers to a chemical moiety, either a nucleotide,
oligonucleotide, polynucleotide or an amino acid,
peptide or protein or other chemical, that when added to
another sequence, provides additional utility or confers
15 useful properties, particularly in the detection or
isolation, to that sequence. Thus, for example, a
homopolymer nucleic acid sequence or a nucleic acid
sequence complementary to a capture oligonucleotide may
be added to a primer or probe sequence to facilitate the
20 subsequent isolation of an extension product or
hybridized product. In the case of protein tags,
histidine residues (e.g., 4 to 8 consecutive histidine
residues) may be added to either the amino- or
carboxy-terminus of a protein to facilitate protein
25 isolation by chelating metal chromatography.
Alternatively, amino acid sequences, peptides, proteins
or fusion partners representing epitopes or binding
determinants reactive with specific antibody molecules
or other molecules (e.g., flag epitope, c-myc epitope,
30 transmembrane epitope of the influenza A virus
hemagglutinin protein, protein A, cellulose binding
domain, calmodulin binding protein, maltose binding
protein, chitin binding domain, glutathione
S-transferase, and the like) may be added to proteins to
35 facilitate protein isolation by procedures such as
affinity or immunoaffinity chromatography. Chemical tag
moieties include such molecules as biotin, which may be
added to either nucleic acids or proteins and

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5 facilitates isolation or detection by interaction with
avidin reagents, and the like. Numerous other tag
moieties are known to, and can be envisioned by, the
trained artisan, and are contemplated to be within the
scope of this definition.

10 The terms "transform", "transfect", "transduce",
shall refer to any method or means by which a nucleic
acid is introduced into a cell or host organism and may
be used interchangeably to convey the same meaning.
Such methods include, but are not limited to,
15 transfection, electroporation, microinjection, PEG-
fusion and the like.

The introduced nucleic acid may or may not be
integrated (covalently linked) into nucleic acid of the
recipient cell or organism. In bacterial, yeast, plant
20 and mammalian cells, for example, the introduced nucleic
acid may be maintained as an episomal element or
independent replicon such as a plasmid. Alternatively,
the introduced nucleic acid may become integrated into
the nucleic acid of the recipient cell or organism and
25 be stably maintained in that cell or organism and
further passed on or inherited to progeny cells or
organisms of the recipient cell or organism. In other
manners, the introduced nucleic acid may exist in the
recipient cell or host organism only transiently.

30 A "clone" or "clonal cell population" is a
population of cells derived from a single cell or common
ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell
population that is capable of stable growth *in vitro* for
35 many generations.

An "immune response" signifies any reaction
produced by an antigen, such as a protein antigen, in a
host having a functioning immune system. Immune

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5 responses may be either humoral in nature, that is,
involve production of immunoglobulins or antibodies, or
cellular in nature, involving various types of B and T
lymphocytes, dendritic cells, macrophages, antigen
presenting cells and the like, or both. Immune
10 responses may also involve the production or elaboration
of various effector molecules such as cytokines,
lymphokines and the like. Immune responses may be
measured both in *in vitro* and in various cellular or
animal systems.

15 An "antibody" or "antibody molecule" is any
immunoglobulin, including antibodies and fragments
thereof, that binds to a specific antigen. The term
includes polyclonal, monoclonal, chimeric, and
bispecific antibodies. As used herein, antibody or
20 antibody molecule contemplates both an intact
immunoglobulin molecule and an immunologically active
portion of an immunoglobulin molecule such as those
portions known in the art as Fab, Fab', F(ab')₂ and
F(v).

25

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A - 1D depict SDS polyacrylamide gel
analysis of purified CEL I and CEL II. Fig. 1A: Lane 1,
molecular weight standards shown in kDa on the side.
30 Lane 2, 1 µg of homogeneous CEL I enzyme. Panels B and C
examine the mobility changes in the CEL I and CEL II
protein bands due to EndoH_f treatment. Samples in panel B
contain only CEL I. Samples in Panel C contain a mixture
of CEL I and CEL II. Panel D shows the mobility change
35 of homogeneous CEL I after sulfhydryl reduction. The
gels were stained with Gelcode Blue. Fig. 1B: Lane 1,
Endo H_f. Lane 2: molecular weight standards. Lane 3,
homogeneous CEL I, about 30 ng. Lane 4, CEL I digested

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5 with Endo H_f. Fig. 1C: Lane 1, Endo H_f. Lane 2:
molecular weight standards. Lane 3, Purified CEL I with
a small amount of CEL II. Lane 4, CEL I and CEL II
digested with Endo H_f. Fig. 1D: Purified CEL I was
10 boiled for 2 min in SDS sample buffer in the presence
(lane 2) or absence (lane 3) of 1% β -mercaptoethanol.
Lane 1: molecular weight standards. H = Endo H_f, I = CEL
I, II = CEL II.

Figure 2 shows the cDNA (SEQ ID NO: 1) and amino
15 acid sequence (SEQ ID NO: 2) of CEL I. The amino acid
sequences of CEL I determined by Edman degradation are
shown in bold. They consist of the N-terminal sequence:
WSKEGHVMTQCIAQDLLEPEAAHAVKMLLPDYANGXLSSLXVWP; internal
peptide from GluC digest: XSWLQDVE; internal peptides
20 from tryptic digest: CDDISTCANKYAKE and LACNWGYK. The
residues identical with DSA6, BFN1 and ZEN1 are
underlined. The conserved cys residues are shown with #
underneath. The nine conserved residues shown to be
ligands for the three Zn atoms in P1 nuclease are shown
25 with + underneath.

Figure 3 is a picture of a gel image of mutation
detection analyses on a Perkin Elmer automated DNA
sequencer running the GeneScan program showing the
30 effects of Mg⁺⁺ and pH on CEL I mutation detection. The
substrate is a 235 bp PCR product of the *BRCA1* gene exon
5 containing a T-G polymorphism. It is labeled at the
5' terminus with 6-FAM (Blue) in the top strand and with
TET (Green) on the bottom strand. The substrates were
35 incubated with 0.5 units of CEL I for 30 min at 45 °C
and then analyzed as described in Fig. 6. In lane 5 the
band at 156 nt (labeled "blue cut") corresponds to CEL I
mismatch-specific cutting on the 6-FAM-labeled strand,

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5 and the band at 80 nucleotides (labeled "green cut") corresponds to the mismatch-specific cutting on the TET-labeled strand. The bands at the bottom in the gel image show the internal size standards in each lane.

10 Figures 4A-4F show nicking of RF-I DNA by CEL I and mung bean nuclease. Assays are in the presence (solid symbols) or absence (hollow symbols) of 3 mM MgCl_2 . Panels A, C, and E are assays at pH 5.5. Panels B, D, and F are at pH 7.5.

15 Figures 5A-5C show the solubilization of denatured calf-thymus DNA by CEL I and mung bean nuclease. Assays are in the presence (solid symbols) or absence (hollow symbols) of 3 mM MgCl_2 . Circles are assays at pH 5.5. Squares are at pH 7.5. The enzymes tested in panels A, B, and C are MBN-A, MBN-B, and CEL I, respectively. One unit of single-strand nuclease activity of CEL I equals 32 ng of homogeneous CEL I (3.1×10^4 single-strand nuclease units/mg enzyme as seen in initial kinetics up to 20 min in panel C).

Figures 6A-6I are electropherograms comparing mismatch detection mediated by CEL I and MBN. Electropherograms of Genescan fragment analysis on an PE-Biosystems automated DNA sequencer are shown. Two color fluorescent heteroduplexes of PCR products of *BRCA1* gene were prepared as described in the experimental procedures. Vertical axis, relative fluorescence units; horizontal axis, DNA length in nucleotides. In Panels A, D, and G, the DNA was incubated with 7 ng of MBN-A. In Panels B, E, and H, the DNA was incubated with 11 ng of MBN-B. In Panels C, F, and I, the DNA was incubated with 10 pg of CEL I. These

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5 reactions were performed in Buffer I with 3 mM MgCl₂ for
30 min at 37 C. In panels A, B, and C, the substrate was
a 387 bp heteroduplex containing a 4 nt deletion. In
panels D, E, and F, the substrate was a 323 bp product
containing a C-T base substitution mismatch. In panels
10 G, H, and I, the substrate was a 402 bp heteroduplex
containing a C insertion in one strand. In each of
panels A, B, and C the peak at 129 nt corresponds to
cutting at the 4 base insertion on the 6-FAM-labeled
strand; the peak at 258 nt corresponds to the cutting at
15 the 4 base insertion on the TET-labeled strand. In
panels D, E, G, and H, no mismatch-specific cutting is
seen by the two MBN's. In panel F, the peak at 183 nt
corresponds to CEL I-mismatch-specific cutting on the 6-
FAM-labeled strand, and the peak at 142 nt corresponds
20 to the mismatch-specific cutting on the TET-labeled
strand. In panel I, the peak at 252 nt corresponds to
the CEL I specific cutting at the extrahelical G on the
TET-labeled strand.

25 Fig. 7 shows the solubilization of RNA by CEL I and
mung bean nuclease. Torula yeast RNA was incubated with
0.7 ng of MBN-1 (solid circles) or 16 ng of CEL I
(hollow circles) in the presence of 3 mM MgCl₂ at pH 5.5
(A) and pH 7.5 (B).

30 Fig. 8 is a gel showing polyacrylamide gel analysis
of the CEL I purification fractions. Aliquots of CEL I
with approximately equal amounts of CEL I activity from
each step of enzyme purification was boiled in SDS gel
35 buffer in the absence of reducing agents, and resolved
on a SDS polyacrylamide gel as detailed in the
experimental procedures. The nucleases, after
renaturation, digested the denatured DNA embedded in the

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5 gel. The undigested DNA was stained with Toluidine Blue
0 to provide a negative image of the positions of the
nucleases. Lane 1: molecular weight markers; Lane 2:
buffered celery juice; Lane 3: 25% ammonium sulfate
fractionation supernatant; Lane 4: 80% ammonium sulfate
10 fractionation pellet; Lane 5: sample to ConA Sepharose
column; Lane 6: eluate from ConA Sepharose column; Lane
7: eluate from DEAE-Sepharose column; Lane 8: eluate from
Phosphocellulose P-11 column; Lane 9, eluate from Phenol
Sepharose column; Lane 10: pool of fractions 11 and 12
15 from Mono Q column.

Figs. 9A and 9B are a pair of gels showing incision
at mismatch substrate by CEL I, CEL II proteins
renatured from SDS gel, before and after removal of
20 carbohydrate moieties. CEL I and CEL II protein bands
were excised from a SDS gel and renatured as described
in experimental procedures. The renatured enzyme was
used to digest a 402 bp fluorescently labeled PCR
product of exon 20 of the BRCA 1 gene. Lanes 1-6 are
25 homoduplexes made from wild-type DNA samples containing
no mismatch in exon 20. Lanes 7-12, because of the
heterozygous nature of this sequence in the sample, the
PCR product is a heteroduplex in which one strand
contains a G residue insertion. Cel I incision at the
30 3' side of the extrahelical G residue produces a green
band, indicated in the figure as "incision at inserted
G." Lanes 1 and 7: substrate with no CEL I treatment;
Lanes 2 and 8: incision of the substrate by purified
native CEL I; Lanes 3 and 9: incision of substrate by
renatured 29 KDa CEL I polypeptide band originated from
35 EndoH_f digestion of the 43 KDa CEL I band; Lanes 4 and
10: incision of the substrate by the renatured 37 KDa
CEL II polypeptide band originated from EndoH_f digestion

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5 of the 39 KDa CEL II band; Lanes 5, 6, 11, and 12:
incision of the substrate by renatured 43 KDa CEL I
band.

Fig. 10 shows a Clustal W alignment of CEL I amino
10 acid sequence with homologous sequences. The Genbank
accession numbers of the homologous sequences are
indicated in brackets. 1: (P24021) nuclease S1 of
Aspergillus oryzae; 2: (P24289) nuclease P1 of
Penicillium citrinum; 3 CEL I cDNA amino acid sequence;
15 4: (ABO03131) ZEN 1 endonuclease from *Zinnia elegans*; 5:
(AF082031) daylily senescence-associated protein 6
(DSA6) of *Hermocallis hybrid cultivar*; 6: (U90264)
bifunctional nuclease BFN 1 of *Arabidopsis thaliana*.
ClustalW Multiple Sequence Alignment Kim C. Worley,
20 Human Genome Center- Baylor College of Medicine.
(<http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/multi-align/multi-align.pl>). The secondary structure of P1
nuclease (Volbeda, A., Lahm, A., Sakiyama, F., and Suck,
D., EMBO J. (1991) 10:1607-1618 and Romier, C.,
25 Dominguez, R., Lahm, A., Dahl, O., and Suck, D., (1998)
Proteins: Structure, Function and Genetics 32: 414-424)
is indicated. The nine residues that bind the three Zn
atoms are indicated in bold. The corresponding Zn atoms
to these residues are indicated beneath the alignment.

30

DETAILED DESCRIPTION OF THE INVENTION

The enzymatic basis for the maintenance of correct
base sequences during DNA replication has been
extensively studied in *E. coli*. This organism has
35 evolved a mismatch repair pathway that corrects a
variety of DNA basepair mismatches in hemimethylated DNA
as well as insertions/deletions up to four nucleotides
long. Cells deficient in this pathway mutate more

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5 frequently, hence the genes are called MutS, MutL and
MutH etc. MutS protein binds to the mismatch and MutH
is the endonuclease that incises the DNA at a GATC site
on the strand in which the A residue is not methylated.
MutL forms a complex with MutH and MutS during repair.
10 Homologs of MutS and MutL, but not MutH exist in many
systems. In yeast MSH2 (MutS homolog) can bind to a
mismatch by itself, but a complex of two MutL homologs
(MLH and PMS1) plus a MSH2 has been observed. The
human homolog hMSH2 has evolved to bind to larger DNA
15 insertions up to 14 nucleotides in length, which
frequently arise by mechanisms such as misalignment at
the microsatellite repeats in humans. Mutations in any
one of these human homologs were shown to be responsible
for the hereditary form of non-polyposis colon cancer
20 (27, 28).

Celery contains over 40 µg of psoralen, a
photoreactive intercalator, per gram of tissue (3). As
a necessity, celery may possess a high capability for
the repair of lesions of insertion, deletion, and other
25 psoralen photoadducts. Single-strandedness at the site
of the lesion is common to base substitution and DNA
loop lesions. The data in the following examples
demonstrate that celery possesses nucleic acid sequences
which encode ample mismatch-specific endonuclease to
30 deal with these potentially mutagenic events.

CEL I, isolated from celery, is the first
eukaryotic nuclease known that cleaves DNA with high
specificity at sites of base-substitution mismatch and
DNA distortion. The enzyme requires Mg⁺⁺ and Zn⁺⁺ for
35 activity, with pH optimum at neutral pH. We have
purified CEL I 33,000 fold to apparent homogeneity. A
key improvement is the use of α-methyl-mannoside in the
purification buffers to overcome the aggregation of

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5 glycoproteins with endogenous lectins. The SDS gel electrophoresis band for the homogeneous CEL I, with and without the removal of its carbohydrate moieties, was extracted, renatured, and shown to have mismatch cutting specificity. After the determination of amino acid
10 sequence of 28% of the CEL I polypeptide, we cloned the CEL I cDNA. Potential orthologs are nucleases putatively encoded by the genes *BFN1* of *Arabidopsis*, *ZEN1* of *Zinnia*, and *DSA6* of daylily. Homology of CEL I with S1 and P1 nucleases are much lower. The nuclease
15 activities of CEL I were characterized in comparison to the mung bean nuclease, the closest plant ortholog of S1 nuclease, to establish that these enzymes are catalytically distinct. Single-strandedness in a mismatch substrate does not appear to be the major
20 feature recognized by CEL I. We propose that CEL I exemplifies a new family of neutral pH optimum, magnesium-stimulated, mismatch duplex-recognizing nucleases, within the S1 superfamily.

25 **I. Preparation of CEL I-Encoding Nucleic Acid Molecules, CEL I Proteins, and Antibodies Thereto**

A. Nucleic Acid Molecules

Nucleic acid molecules encoding the CEL I endonuclease of the invention may be prepared by two
30 general methods: (1) Synthesis from appropriate nucleotide triphosphates; or (2) Isolation from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence
35 information, such as the nearly full length cDNA having Sequence I.D. No. 1, enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic oligonucleotides

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5 may be prepared by the phosphoramidite method employed
in the Applied Biosystems 380A DNA Synthesizer or
similar devices. The resultant construct may be
purified according to methods known in the art, such as
high performance liquid chromatography (HPLC). Long,
10 double-stranded polynucleotides, such as a DNA molecule
of the present invention, must be synthesized in stages,
due to the size limitations inherent in current
oligonucleotide synthetic methods. Thus, for example, a
2.4 kb double-stranded molecule may be synthesized as
15 several smaller segments of appropriate complementarity.
Complementary segments thus produced may be annealed
such that each segment possesses appropriate cohesive
termini for attachment of an adjacent segment. Adjacent
segments may be ligated by annealing cohesive termini in
20 the presence of DNA ligase to construct an entire 2.4 kb
double-stranded molecule. A synthetic DNA molecule so
constructed may then be cloned and amplified in an
appropriate vector. Nucleic acid sequences encoding CEL
I may be isolated from appropriate biological sources
25 using methods known in the art. In a preferred
embodiment, a cDNA clone is isolated from a cDNA
expression library of celery origin. In an alternative
embodiment, utilizing the sequence information provided
by the cDNA sequence, genomic clones encoding CEL I may
30 be isolated. Alternatively, cDNA or genomic clones
having homology with CEL I may be isolated from other
plant species, using oligonucleotide probes
corresponding to predetermined sequences within the CEL
I gene.

35 In accordance with the present invention, nucleic
acids having the appropriate level of sequence homology
with the protein coding region of Sequence I.D. No. 1
may be identified by using hybridization and washing

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5 conditions of appropriate stringency. For example, hybridizations may be performed using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to .50%
10 formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 0.5-1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1
15 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between
20 nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989):
$$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\#\text{bp in duplex}$$

As an illustration of the above formula, using
25 $[\text{Na}^+] = [0.368]$ and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be
30 observed using a hybridization temperature of 42°C. Such a sequence would be considered substantially homologous to the nucleic acid sequence of the present invention.

As can be seen from the above, the stringency of
35 the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the two nucleic acid molecules, the hybridization is usually

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5 carried out at salt and temperature conditions that are 20-25°C below the calculated T_m of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be

10 approximately 12-20°C below the T_m of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C,

15 and wash in 2X SSC and 0.5% SDS at 55°C for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and wash in 1X SSC and 0.5% SDS at 65°C for 15 minutes.

20 A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 42°C, and wash in 0.1X SSC and 0.5% SDS at 65°C for 15 minutes.

25 Nucleic acids of the present invention may be maintained as DNA in any convenient cloning vector. In a preferred embodiment, clones are maintained in a plasmid cloning/expression vector, such as pBluescript (Stratagene, La Jolla, CA), which is propagated in a

30 suitable *E. coli* host cell. Genomic clones of the invention encoding the CEL I gene may be maintained in lambda phage FIX II (Stratagene).

CEL I-encoding nucleic acid molecules of the invention include cDNA, genomic DNA, RNA, and fragments

35 thereof which may be single- or double-stranded. Thus, this invention provides oligonucleotides (sense or antisense strands of DNA or RNA) having sequences capable of hybridizing with at least one sequence of a

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5 nucleic acid molecule of the present invention, such as
selected segments of the cDNA having Sequence I.D. No.
1. Such oligonucleotides are useful as probes for
detecting or isolating CEL I genes.

10 It will be appreciated by persons skilled in the
art that variants (e.g., allelic variants) of these
sequences exist in the celery population, and must be
taken into account when designing and/or utilizing
oligos of the invention. Accordingly, it is within the
scope of the present invention to encompass such
15 variants, with respect to the CEL I sequences disclosed
herein or the oligos targeted to specific locations on
the respective genes or RNA transcripts. With respect
to the inclusion of such variants, the term "natural
allelic variants" is used herein to refer to various
20 specific nucleotide sequences and variants thereof that
would occur in a given DNA population. Genetic
polymorphisms giving rise to conservative or neutral
amino acid substitutions in the encoded protein are
examples of such variants. Additionally, the term
25 "substantially complementary" refers to oligo sequences
that may not be perfectly matched to a target sequence,
but the mismatches do not materially affect the ability
of the oligo to hybridize with its target sequence under
the conditions described.

30 Thus, the coding sequence may be that shown in
Sequence I.D. No. 1, or it may be a mutant, variant,
derivative or allele of this sequence. The sequence may
differ from that shown by a change which is one or more
of addition, insertion, deletion and substitution of one
35 or more nucleotides of the sequence shown. Changes to a
nucleotide sequence may result in an amino acid change
at the protein level, or not, as determined by the
genetic code.

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5 Thus, nucleic acid according to the present invention may include a sequence different from the sequence shown in Sequence I.D. No. 1 yet encode a polypeptide with the same amino acid sequence.

10 On the other hand, the encoded polypeptide may comprise an amino acid sequence which differs by one or more amino acid residues from the amino acid sequence shown in Sequence I.D. No. 2. Nucleic acid encoding a polypeptide which is an amino acid sequence mutant, variant, derivative or allele of the sequence shown in
15 Sequence I.D. No. 2 is further provided by the present invention. Nucleic acid encoding such a polypeptide may show greater than 60% identity with the coding sequence shown in Sequence I.D. No. 1, greater than about 70% identity, greater than about 80% identity, greater than
20 about 90% identity or greater than about 95% identity.

 The present invention provides a method of obtaining nucleic acid of interest, the method including hybridization of a probe having part or all of the sequence shown in Sequence I.D. No. 1 or a complementary
25 sequence, to target nucleic acid. Hybridization is generally followed by identification of successful hybridization and isolation of nucleic acid which has hybridized to the probe, which may involve one or more steps of PCR.

30 Such oligonucleotide probes or primers, as well as the full-length sequence (and mutants, alleles, variants, and derivatives) are useful in screening a test sample containing nucleic acid for the presence of alleles, mutants or variants of CEL I, the probes
35 hybridizing with a target sequence from a sample obtained from a plant being tested. The conditions of the hybridization can be controlled to minimize non-specific binding, and preferably stringent to moderately

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5 stringent hybridization conditions are used. The skilled person is readily able to design such probes, label them and devise suitable conditions for hybridization reactions, assisted by textbooks such as Sambrook et al (1989) and Ausubel et al (1992).

10 In some preferred embodiments, oligonucleotides according to the present invention that are fragments of the sequences shown in Sequence I.D. No. 1 or any allele associated with endonuclease activity, are at least about 10 nucleotides in length, more preferably at least
15 15 nucleotides in length, more preferably at least about 20 nucleotides in length. Such fragments themselves individually represent aspects of the present invention. Fragments and other oligonucleotides may be used as primers or probes as discussed but may also be generated
20 (e.g. by PCR) in methods concerned with determining the presence in a test sample of a sequence encoding a homolog of CEL I nuclease.

B. Proteins

25 CEL I is the first eucaryotic nuclease identified which cleaves DNA with high specificity at sites of base-substitution mismatch and DNA distortion. A full-length CEL I protein of the present invention may be prepared in a variety of ways, according to known
30 methods. The protein may be purified from appropriate sources, e.g., from celery as described in US Patent 5,869,245, the entire disclosure of which is incorporated by reference herein. However, this is not a preferred method due to the low amount of protein
35 likely to be present in a given cell type at any time. The availability of nucleic acid molecules encoding CEL I enables production of the protein using *in vitro* expression methods known in the art. For example, a

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5 cDNA or gene may be cloned into an appropriate *in vitro*
transcription vector, such as pSP64 or pSP65 for *in*
vitro transcription, followed by cell-free translation
in a suitable cell-free translation system, such as
wheat germ or rabbit reticulocyte lysates. *In vitro*
10 transcription and translation systems are commercially
available, e.g., from Promega Biotech, Madison,
Wisconsin or BRL, Rockville, Maryland.

Alternatively, according to a preferred
embodiment, larger quantities of CEL I may be produced
15 by expression in a suitable prokaryotic or eukaryotic
system. For example, part or all of a DNA molecule,
such as the cDNA having Sequence I.D. No. 1, may be
inserted into a plasmid vector adapted for expression in
a bacterial cell, such as *E. coli*. Such vectors
20 comprise the regulatory elements necessary for
expression of the DNA in the host cell (e.g. *E. coli*)
positioned in such a manner as to permit expression of
the DNA in the host cell. Such regulatory elements
required for expression include promoter sequences,
25 transcription initiation sequences and, optionally,
enhancer sequences.

The CEL I produced by gene expression in a
recombinant prokaryotic or eukaryotic system may be
purified according to methods known in the art. In a
30 preferred embodiment, a commercially available
expression/secretion system can be used, whereby the
recombinant protein is expressed and thereafter secreted
from the host cell, to be easily purified from the
surrounding medium. If expression/secretion vectors are
35 not used, an alternative approach involves purifying the
recombinant protein by affinity separation, such as by
immunological interaction with antibodies that bind
specifically to the recombinant protein or nickel

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5 columns for isolation of recombinant proteins tagged with 6-8 histidine residues at their N-terminus or C-terminus. Alternative tags may comprise the FLAG epitope or the hemagglutinin epitope. Such methods are commonly used by skilled practitioners.

10 The CEL I proteins of the invention, prepared by the aforementioned methods, may be analyzed according to standard procedures. For example, such proteins may be subjected to amino acid sequence analysis, according to known methods.

15 Polypeptides which are amino acid sequence variants, alleles, derivatives or mutants are also provided by the present invention. A polypeptide which is a variant, allele, derivative, or mutant may have an amino acid sequence that differs from that given in
20 Sequence I.D. No. 2 by one or more of addition, substitution, deletion and insertion of one or more amino acids. Preferred such polypeptides have CEL I function, that is to say have one or more of the following properties: ability to cleave mismatched
25 heteroduplex DNA; immunological cross-reactivity with an antibody reactive with the polypeptide for which the sequence is given in Sequence I.D. No. 2; sharing an epitope with the polypeptide for which the sequence is given in Sequence I.D. No. 2 (as determined for example
30 by immunological cross-reactivity between the two polypeptides.

A polypeptide which is an amino acid sequence variant, allele, derivative or mutant of the amino acid sequence shown in Sequence I.D. No. 2 may comprise an
35 amino acid sequence which shares greater than about 35% sequence identity with the sequence shown, greater than about 40%, greater than about 50%, greater than about 60%, greater than about 70%, greater than about 80%,

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5 greater than about 90% or greater than about 95%.
Particular amino acid sequence variants may differ from
that shown in Sequence I.D. No.2 by insertion, addition,
substitution or deletion of 1 amino acid, 2, 3, 4, 5-10,
10-20, 20-30, 30-40, 40-50, 50-100, 100-150, or more
10 than 150 amino acids. For amino acid "homology", this
may be understood to be identity or similarity
(according to the established principles of amino acid
similarity, e.g., as determined using the algorithm GAP
(Genetics Computer Group, Madison, WI). GAP uses the
15 Needleman and Wunsch algorithm to align two complete
sequences that maximizes the number of matches and
minimizes the number of gaps. Generally, the default
parameters are used, with a gap creation penalty = 12
and gap extension penalty = 4. Use of GAP may be
20 preferred but other algorithms may be used including
without limitation, BLAST (Altschul et al. (1990 J. Mol.
Biol. 215:405-410); FASTA (Pearson and Lipman (1998)
PNAS USA 85:2444-2448) or the Smith Waterman algorithm
(Smith and Waterman (1981) J. Mol. Biol. 147:195-197)
25 generally employing default parameters. Use of either
of the terms "homology" and "homologous" herein does not
imply any necessary evolutionary relationship between
the compared sequences. The terms are used as they are
in the phrase "homologous recombination", i.e., the
30 terms merely require that the two nucleotide sequences
are sufficiently similar to recombine under appropriate
conditions.

A polypeptide according to the present
invention may be used in screening for molecules which
35 affect or modulate its activity or function. Such
molecules may be useful for research purposes.

The present invention also provides antibodies
capable of immunospecifically binding to proteins of the

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5 invention. Polyclonal antibodies directed toward CEL I
may be prepared according to standard methods. In a
preferred embodiment, monoclonal antibodies are
prepared, which react immunospecifically with various
epitopes of CEL I. Monoclonal antibodies may be
10 prepared according to general methods of Köhler and
Milstein, following standard protocols. Polyclonal or
monoclonal antibodies that immunospecifically interact
with CEL I can be utilized for identifying and purifying
such proteins. For example, antibodies may be utilized
15 for affinity separation of proteins with which they
immunospecifically interact. Antibodies may also be
used to immunoprecipitate proteins from a sample
containing a mixture of proteins and other biological
molecules. Other uses of anti-CEL I antibodies are
20 described below.

Antibodies according to the present invention may
be modified in a number of ways. Indeed the term
"antibody" should be construed as covering any binding
substance having a binding domain with the required
25 specificity. Thus, the invention covers antibody
fragments, derivatives, functional equivalents and
homologues of antibodies, including synthetic molecules
and molecules whose shape mimics that of an antibody
enabling it to bind an antigen or epitope.

30 Exemplary antibody fragments, capable of binding an
antigen or other binding partner, are Fab fragment
consisting of the VL, VH, C1 and CH1 domains; the Fd
fragment consisting of the VH and CH1 domains; the Fv
fragment consisting of the VL and VH domains of a single
35 arm of an antibody; the dAb fragment which consists of a
VH domain; isolated CDR regions and F(ab')₂ fragments, a
bivalent fragment including two Fab fragments linked by

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5 a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

II. Uses of CEL I-Encoding Nucleic Acids, CEL I Proteins and Antibodies Thereto

10 CEL I appears to be an DNA endonuclease which may be used to advantage in mutational screening assays. Specifically, the CEL I molecules of the invention may be used to advantage in genetic screening assays to identify those patients that may be at risk for certain
15 genetic disorders. Such disorders include, without limitation, sickle cell anemia, cystic fibrosis, lysosomal storage diseases and genetic mutations that predispose a patient to cancer.

Additionally, CEL I nucleic acids, proteins and
20 antibodies thereto, according to this invention, may be used as a research tool to identify other proteins that are intimately involved in DNA recognition and repair reactions. Biochemical elucidation of the DNA recognition and repair capacity of CEL I will facilitate
25 the development of these novel screening assays for assessing a patient's propensity for cancer and genetic disease.

30

A. CEL I-Encoding Nucleic Acids

CEL I-encoding nucleic acids may be used for a variety of purposes in accordance with the present invention. CEL I-encoding DNA, RNA, or fragments
35 thereof may be used as probes to detect the presence of and/or expression of genes encoding CEL I-like proteins. Methods in which CEL I-encoding nucleic acids may be utilized as probes for such assays include, but are not

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5 limited to: (1) *in situ* hybridization; (2) Southern hybridization (3) northern hybridization; and (4) assorted amplification reactions such as polymerase chain reactions (PCR).

10 The CEL I-encoding nucleic acids of the invention may also be utilized as probes to identify related genes from other plant and animal species. As is well known in the art, hybridization stringencies may be adjusted to allow hybridization of nucleic acid probes with complementary sequences of varying degrees
15 of homology. Thus, CEL I-encoding nucleic acids may be used to advantage to identify and characterize other genes of varying degrees of relation to CEL I, thereby enabling further characterization of the DNA mismatch recognition system. Additionally, they may be used to
20 identify genes encoding proteins that interact with CEL I (e.g., by the "interaction trap" technique), which should further accelerate identification of the components involved in DNA mismatch recognition.

Nucleic acid molecules, or fragments thereof,
25 encoding CEL I may also be utilized to control the production of CEL I, thereby regulating the amount of protein available to participate in DNA mismatch recognition reactions. Alterations in the physiological amount of CEL I protein may dramatically affect the
30 activity of other protein factors involved in DNA mismatch recognition.

The availability of CEL I encoding nucleic acids enables the production of strains of laboratory mice carrying part or all of the CEL I gene or mutated
35 sequences thereof. Such mice may provide an *in vivo* model for assessing CEL I activity in a mammalian milieu. Methods of introducing transgenes in laboratory mice are known to those of skill in the art. Three

- 40 -

5 common methods include: 1. integration of retroviral
vectors encoding the foreign gene of interest into an
early embryo; 2. injection of DNA into the pronucleus
of a newly fertilized egg; and 3. the incorporation of
genetically manipulated embryonic stem cells into an
10 early embryo. Production of the transgenic mice
described above will facilitate the molecular elucidation
of the role CEL I plays in DNA mismatch recognition.

The term "animal" is used herein to include all
vertebrate animals, except humans. It also includes an
15 individual animal in all stages of development,
including embryonic and fetal stages. A "transgenic
animal" is any animal containing one or more cells
bearing genetic information altered or received,
directly or indirectly, by deliberate genetic
20 manipulation at the subcellular level, such as by
targeted recombination or microinjection or infection
with recombinant virus. The term "transgenic animal" is
not meant to encompass classical cross-breeding or in
vitro fertilization, but rather is meant to encompass
25 animals in which one or more cells are altered by or
receive a recombinant DNA molecule. This molecule may
be specifically targeted to a defined genetic locus, be
randomly integrated within a chromosome, or it may be
extrachromosomally replicating DNA. The term "germ cell
30 line transgenic animal" refers to a transgenic animal in
which the genetic alteration or genetic information was
introduced into a germ line cell, thereby conferring the
ability to transfer the genetic information to
offspring. If such offspring, in fact, possess some or
35 all of that alteration or genetic information, then
they, too, are transgenic animals.

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5 **B. CEL I Protein and Antibodies**

Purified CEL I protein, or fragments thereof, produced via expression of the CEL I encoding nucleic acids of the present invention may be used to produce polyclonal or monoclonal antibodies which also may serve
10 as sensitive detection reagents for the presence and accumulation of CEL I (or complexes containing CEL I) in plant cells. Recombinant techniques enable expression of fusion proteins containing part or all of the CEL I protein. The full length protein or fragments of the
15 protein may be used to advantage to generate an array of monoclonal antibodies specific for various epitopes of the protein, thereby providing even greater sensitivity for detection of the protein in cells.

Polyclonal or monoclonal antibodies
20 immunologically specific for CEL I may be used in a variety of assays designed to detect and quantitate the protein. Such assays include, but are not limited to: (1) flow cytometric analysis; (2) immunochemical localization of CEL I in plant cells; and (3) immunoblot
25 analysis (e.g., dot blot, Western blot) of extracts from various cells. Additionally, as described above, anti-CEL I can be used for purification of CEL I and orthologs thereof (e.g., affinity column purification, immunoprecipitation).

30 CEL I protein may also be used to advantage in genetic screening assays as discussed above.

From the foregoing discussion, it can be seen that CEL I-encoding nucleic acids, CEL I expressing vectors, and anti-CEL I antibodies of the invention can
35 be used to produce large quantities of CEL I protein, detect CEL I gene expression and alter CEL I protein accumulation for purposes of assessing the genetic and

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5 protein interactions involved in the recognition of DNA damage.

The following protocols are provided to facilitate the practice of the present invention.

10 Plasmid DNA pUC19 was isolated with the QIAGEN Maxi Kit from DH5 host cells, following the manufacturer's instructions. Calf thymus DNA was obtained from Sigma and purified by repeated cycles of proteinase K digestion and phenol extraction (9). Chromatography
15 resins and columns were purchased from Pharmacia Biotech. Toluidine Blue O and Ponceau S were from Sigma. Endo H_f was from New England Biolabs. Phosphocellulose P11 was from Whatman.

20 *Purification of CEL I*

All steps were performed at 4 °C. The nuclease activity was monitored by using a RF-I (Replicative Form I) nicking assay (10).

Step 1: Preparation of the crude extract - 105
25 kilograms of chilled celery stalks were homogenized with a juice extractor. The juice was collected (total 79.34 L) and adjusted to the composition of Buffer A (100 mM Tris-HCl, pH 7.7, 100 µM PMSF). Solid (NH₄)₂SO₄ was slowly and gently stirred into the juice, to a final
30 concentration of 25% saturation. After 30 minutes, the suspension was centrifuged at 27,000 x g for 1.5 hours. The supernatant (total 70.56 L) was pooled and the concentration of (NH₄)₂SO₄ was adjusted to 80% saturation. After 30 minutes of stirring, the mixture was
35 centrifuged at 27,000 x g for 2 hours. The pellets were resuspended in Buffer B (0.1 M Tris-HCl, pH 7.7, 0.5 M KCl, 100 µM PMSF) and thoroughly dialyzed against Buffer B.

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5 Step 2: Concanavalin A-Sepharose 4B affinity
chromatography - 100 ml of ConA resin (cross-linked with
dimethylsuberimidate) was added to the 7.71 L sample in
bottles that were gently rolled overnight. The resin was
packed into a 2.5 cm diameter column. The flow-through
10 fraction, containing no CEL I activity, was discarded.
CEL I was eluted at 4 °C by 200 ml of Buffer B
containing 0.3 M α -methyl-mannoside. The elution step
was repeated 10 more times until no more nuclease
activity could be eluted. The eluate was combined and
15 dialyzed against Buffer C (50 mM Tris-HCl, pH 8.0, 5 mM
 α -methyl-mannoside, 0.01% Triton X-100, and 100 μ M
PMSF).

 Step 3: DEAE-Sepharose chromatography - The dialyzed
sample from step 2 (total 2.5 L) was applied to a 400 ml
20 DEAE-Sepharose column of 5 cm diameter previously
equilibrated with Buffer C. The subsequent steps were
performed using FPLC. The column was washed with 400 ml
of Buffer C. CEL I was eluted with a 1 L linear gradient
of 10 mM to 1 M KCl in Buffer C containing 50 mM α -
25 methyl-mannoside at a flow rate of 5 ml/min, followed by
400 ml of Buffer C containing 1 M KCl and 50 mM α -
methyl-mannoside at a flow rate of 8 ml/min. The most
active CEL I fractions were pooled and dialyzed against
Buffer D (25 mM potassium phosphate, pH 7.0, 5 mM α -
30 methyl-mannoside, 0.01 % Triton X-100, and 100 μ M PMSF).

 Step 4: Phosphocellulose P-11 chromatography - The
dialyzed CEL I pool from step 3 (120 ml) was applied to
a 5 cm diameter column packed with 400 ml of P-11 resin.
The column was previously equilibrated with Buffer D at
35 a flow rate of 5 ml/min. After sample loading, the
column was washed with 625 ml of Buffer D containing 50
mM α -methyl-mannoside at a flow rate of 5 ml/min. CEL I
was eluted with a 800 ml linear gradient of 20 mM KCl to

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5 1 M KCl in Buffer D containing 50 mM α -methyl-mannoside
at a flow rate of 5 ml/min. The column was further
washed with 400 ml of Buffer D containing 1 M KCl and 50
mM α -methyl-mannoside at a flow rate of 8 ml/min. The
most active fractions were pooled and dialyzed against
10 Buffer E (50 mM potassium phosphate, pH 7.0, 5 mM α -
methyl-mannoside, 0.01 % Triton X-100, and 100 μ M PMSF)
containing 1.5 M $(\text{NH}_4)_2\text{SO}_4$.

Step 5: Phenyl Sepharose CL-4B chromatography - The
dialyzed CEL I pool from step 4 (480 ml) was applied to
15 a 5 cm diameter column packed with 400 ml of Phenyl
Sepharose CL-4B. The column was previously equilibrated
with Buffer E containing 1.5 M $(\text{NH}_4)_2\text{SO}_4$ at a flow rate of
5 ml/min. After sample application, the column was
washed with 400 ml of Buffer E containing 1.5 M $(\text{NH}_4)_2\text{SO}_4$
20 and 50 mM α -methyl-mannoside at a flow rate of 5 ml/min.
CEL I was eluted from the column with a 500 ml linear
reversed salt gradient from 1.5 M to 0 M $(\text{NH}_4)_2\text{SO}_4$ in
Buffer E containing 50 mM α -methyl-mannoside at a flow
rate of 5 ml/min. The most active fractions were pooled
25 and dialyzed against Buffer F (50 mM Tris-HCl, pH 8.0, 5
mM α -methyl-mannoside, 0.01 % Triton X-100, and 100 mM
PMSF).

Step 6: Mono Q anion-exchange chromatography - A
Pharmacia prepacked Mono Q HR 16/10 column was
30 thoroughly washed and equilibrated with Buffer F. The
dialyzed CEL I pool from step 5 (336 ml) was applied at
a flow rate of 5 ml/min followed by 100 ml of Buffer F
containing 50 mM α -methyl-mannoside at a flow rate of 10
ml/min. CEL I was eluted with a 250 ml linear gradient
35 of 0 - 1 M KCl in Buffer F containing 50 mM α -methyl-
mannoside at 2 ml/min.

Step 7: Superdex 75 size-exclusion chromatography
using the SMART system - The active fractions of step 6,

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5 fraction 11 and 12, were combined and concentrated by
using Centricon 3 centrifugal concentrators. Aliquots of
the concentrated enzyme were applied to a prepacked
Superdex 75 PC 3.2/30 column equilibrated with Buffer G
(50 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 μ M ZnCl₂, 0.01 %
10 Triton X-100, and 100 μ M PMSF) containing 50 mM α -
methyl-mannoside. Five ml of Buffer G containing 50 mM
 α -methyl-mannoside was used to elute CEL I at a flow
rate of 0.05 ml/min. The purity of the active fractions
was checked by SDS-PAGE. When additional protein bands
15 were present, the fractions were pooled, concentrated,
and purified again using the same size exclusion
chromatography until CEL I reached apparent homogeneity.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

20 Polyacrylamide gel electrophoresis in SDS was carried
out as previously described (11). Protein bands were
detected by using the Gelcode Blue Stain Reagent
(Pierce). Molecular weights of the protein bands were
determined by using the semi-logarithmic plot of the
25 molecular weights of protein standards versus their
relative electrophoretic mobilities. Activity gel assay
was performed essentially as described (12-13).

Endo H_f Removal of N-linked oligosaccharides from CEL I

30 CEL I sample was denatured in 0.5% SDS at 100 °C for
10 min. Appropriate amount of Endo H_f was added and the
reaction was incubated in G5 buffer (50 mM Sodium
Citrate, pH 5.5) at 37 °C overnight.

35 *Renaturation of CEL I from SDS-PAGE*

This method is a modification of a procedure
previously described (13-14). The CEL I fractions were
loaded onto the SDS-PAGE in two consecutive lanes. After

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5 electrophoresis, the gel was split between the two
lanes. One half of the gel was stained with Gelcode Blue
Stain Reagent (Pierce) and then aligned with the other
half that was not stained. The gel slice corresponding
to the CEL I band in the unstained gel was excised and
10 eluted using an AMICON model 57005 electroeluter, for 2
hours at 20 mA per sample, using the elution buffer (50
mM Tris-HCl, pH 7.5, 180 mM NaCl, 0.1% SDS, 0.1 mg/ml
BSA). After elution, the sample was concentrated by
using a Centricon 3 unit. Centrifugation was overnight
15 at 7,000 x g. The volume of the sample was measured and
4 volumes of distilled acetone (-20 °C) was added. The
sample was incubated in dry ice-ethanol bath for 30 min
and then centrifuged at 14,000 x g for 10 min. The
precipitated proteins were washed with a buffer
20 consisting of 20% Dilution and Renaturation Solution (50
mM Tris-HCl, pH 7.5, 10% Glycerol, 100 mM NaCl, 10 ml
MgCl₂, 5 mM CaCl₂, 2 μM ZnCl₂ and 0.1 mg/ml BSA) and 80%
acetone. The sample was precipitated again at 14,000 x g
for 10 min. The supernatant was discarded. The residual
25 acetone was decanted by inverting the tube for 10 min.
The pellet was air dried for at least 10 min. Twenty μl
of Renaturation Solution (6 M Guanidine-HCl, 50 mM Tris-
HCl pH 7.5, 10% Glycerol, 100 mM NaCl, 10 ml MgCl₂, 5 mM
CaCl₂, 2 μM ZnCl₂ and 0.1 mg/ml BSA) was then used to
30 dissolve the pellet. After 20 min of incubation at room
temperature, 1 ml of Dilution and Renaturation Solution
was added and the protein was further renatured at room
temperature for 12 hours.

35 *Mismatch endonuclease assay*

The mismatch endonuclease assay was performed as
previously described (8). Briefly, PCR products were
amplified using genomic DNA from two individuals, one

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5 being wild-type and the other being heterozygous for C
insertion in exon 20 in the *BRCA1* gene. The forward
primer was 5'-labeled with 6-FAM (blue) and the reverse
primer was 5'-labeled with TET (green). The location of
the insert in the *BRCA1* gene is 5382 nt position. The
10 resulting heteroduplexes provide 402 bp PCR products
containing an extrahelical C or an extrahelical G. 50 ng
of the fluorescently labeled substrate was incubated
with CEL I for 30 min at 45 C in a reaction volume of 20
µl in 20 mM HEPES pH 7.5, 10 mM KCl, 3 mM MgCl₂. The
15 reactions were processed as described (8), loaded onto a
denaturing 34 cm well-to-read 6 % polyacrylamide gel on
an ABI 377 DNA Sequencer and analyzed using GeneScan 3.1
software (Perkin-Elmer). The results are displayed as a
gel image.

20

Preparation of the CEL I Sample for Sequencing

The purified CEL I sample was subjected to 10% SDS-
PAGE analysis. After electrophoresis, the protein in the
25 gel was electrophoretically transferred to an Immobilon-
PSQ PVDF membrane by using a Western transfer apparatus
(Novex). The transfer buffer contained 12 mM Trizma
base, 96 mM glycine, and 20% methanol. The transfer
condition was 1 hour at 25V (constant voltage). The
30 membrane was next washed extensively with water, and
stained with Ponceau S. The CEL I band was excised,
destained with water, and sent to the Protein/DNA
Technology Center of Rockefeller University for N-
terminal and internal peptide micro-sequencing by
35 automated Edman degradation reaction. The N-terminal
sequence was determined first (15). The remaining
protein fractions were digested with either Trypsin or

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5 GluC. The digested peptides were purified by HPLC, and sequenced with Edman Degradation (16).

Cloning of the cDNA of the CEL I mRNA

10 Total RNA was prepared from fresh celery using the phenol SDS method for plant RNA preparation (17). First strand cDNA was synthesized using Stratagene's ProStar First Strand RT-PCR kit. Degenerate PCR primers were chosen from the amino acid sequences determined by Edman degradation analysis of the pure CEL I protein, and used
15 to amplify the CEL I cDNA in two segments, using the AmpliTaq DNA polymerase, and cloned in *E. coli* for DNA sequencing. The two fragments provided most of the reading frame of the CEL I protein. Using 5' and 3' RACE methods (Clonetech Marathon cDNA amplification kit), the
20 5' and 3' coding regions and untranslated regions (UTR) of CEL I cDNA were obtained. To confirm the authenticity of the cDNA, two PCR primers were designed, one in the 5' UTR, and one in the 3' UTR. These two primers were used to amplify the CEL I cDNA as one fragment from a
25 fresh preparation of celery RNA, using the high fidelity Pfu DNA polymerase for the amplification. The new sequence was cloned in *E. coli*. The DNA sequence confirmed the authenticity of the previous cDNA sequence except for one nucleotide difference that gives another
30 codon for the same amino acid.

Sources of Mung Bean Nuclease

Mung bean nuclease (MBN) was purchased from Pharmacia Biotech, #27-0912, herein called 'MBN-A', or
35 purified as previously described (18), herein called 'MBN-B'. MBN assay conditions and the measurement of protein concentrations vary in different laboratories and may partially influence the quantitation in this

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5 study. MBN-A is FPLC purified, homogeneous, with a
specific activity of 1.64×10^6 units/mg in the
manufacturer's assay conditions, but 1.42×10^6 units/mg
in our assay conditions. The enzyme exhibits a single-
band in SDS PAGE. MBN-B is an older preparation of the
10 original MBN of Kowalski and has a specific activity of
 4×10^5 units/mg in the assay conditions described
herein. The enzyme appeared as a single band of about 39
KDa on a non-reducing SDS PAGE (data not shown). One
unit of MBN-A single-strand DNase activity equals 0.7 ng
15 of enzyme in our assay.

RF-I nicking assay

1.1 μ g of pPK201/cat (a pUC19 plasmid derivative,
data not shown with pUC19 are similar) was incubated
20 with the designated amount of MBN or CEL I for 30
minutes at 37 °C in a volume of 30 μ l of Buffer H (20 mM
sodium acetate pH 5.5, 10 mM KCl), or Buffer I (20 mM
HEPES pH 7.5, 10 mM KCl) in the presence or absence of 3
mM MgCl₂. To stop the reaction, 5 μ l of stop solution
25 (50 mM Tris-HCl, pH 6.8, 3 % SDS, 4.5 % β -
mercaptoethanol, 30 % glycerol, and 0.001 % Bromophenol
Blue) was added. 24 μ l of the final mixture was loaded
onto a 0.8 % agarose gel. After electrophoresis and
staining with ethidium bromide, a photograph of the gel
30 was taken and the negative was scanned using the IS-1000
Digital Imaging System (Alpha Innotech Corporation). The
RF-I band was quantified using IS-1000 v2.02 software.

Single-strand DNase assay

35 The DNA solubilization assay was similar to that
previously described (19). Fifty μ g of heat-denatured
calf thymus DNA (Calbiochem # 2618, purified by repeated
pronase treatment, phenol extraction and dialysis) was

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5 incubated with 0.7 ng of MBN-A, or 1.9 ng of MBN-B, or
16 ng of CEL I, in 100 μ l of Buffer H or Buffer I, with
or without 3 mM $MgCl_2$. At the designated times, 100 μ l of
cold 20 mM $LaCl_3$ in 0.2 N HCl was added to stop the
reaction. After centrifugation (21,000 x g, 40 min), the
10 absorbance at 260 nm of the supernatant was measured
using a spectrophotometer to determine the amount of DNA
that had become acid-soluble.

Mismatch endonuclease assay

15 The mismatch endonuclease assay was performed as
previously described (8). Briefly, PCR products were
amplified using genomic DNA of individuals that are
heterozygous for certain alterations in three different
exons in the *BRCA1* gene. The forward primer was 5'-
20 labeled with 6-FAM (blue) and the reverse primer was 5'-
labeled with TET (green). The location of the mismatches
in the *BRCA1* gene are 300 nt, 4184 nt, 4421 nt, and 5382
nt positions. They correspond to a T-G base substitution
in exon 5, a 4 base deletion in exon 11, a C-T
25 polymorphism in exon 13, and a C insertion in exon 20,
respectively. The four resulting heteroduplexes provide
a 235 bp PCR product containing a T/C or a G/A base-
substitution mismatch, a 387 bp PCR product containing a
4 base loop, a 323 bp product containing either a C/A or
30 a T/G base-substitution mismatch, and a 402 bp product
containing an extrahelical C or an extrahelical G. 50 ng
of the fluorescently labeled heteroduplex was incubated
with 7 ng of MBN-A, or 11 ng of MBN-B, or 10 pg of CEL I
(0.3 units) for 30 min at 37°C or 45 C in a reaction
35 volume of 20 μ l in Buffer I in the presence or absence
of 3 mM $MgCl_2$. The reactions were processed as described
(8), loaded onto a denaturing 34 cm well-to-read 6 %
polyacrylamide gel on an ABI 377 Sequencer and analyzed

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5 using GeneScan 3.1 software (Perkin-Elmer). The results are displayed as the peak profile of each lane of the gel image (Fig. 6).

10 *Single-Strand RNase assay*

Fifty µg of purified *Torula* Yeast RNA (Amicon #7120) was incubated with 0.7 ng of MBN-A, or 16 ng of CEL I, in 100 µl of Buffer H or Buffer I, with 3 mM MgCl₂ at 37 C. At the designated times, 13 µl of cold 3M sodium acetate pH 5.2 and 282 µl of ethanol was added. The mixture was put at -20 °C overnight. After centrifugation to precipitate the RNA (21,000 x g, 45 min), the absorbance at 260 nm of the supernatant was measured using a spectrophotometer to determine the amount of RNA that had become soluble.

The following examples are provided to describe the invention in further detail. These examples, which set forth the best mode presently contemplated for carrying out the invention, are intended to illustrate and not to limit the invention.

EXAMPLE I

Purification of CEL I

CEL I was purified to homogeneity, more than 33,000 fold over its specific activity in the buffered celery juice. Table 1 summarizes the purification of CEL I from 105 Kg of celery stalks. The active band of CEL I is of the same size throughout purification as judged by an activity gel assay. See Figure 8. There are two nuclease bands that copurify during all the purification steps. We show below that the minor band is not derived from the major band. The major nuclease activity, designated CEL I, migrates at 43 KDa on SDS PAGE (Fig.

- 52 -

5 1A). The minor activity at 39 KDa is a putative isozyme
we named CEL II Fig. 1C, lane 3), also capable of
cutting at mismatches.

10

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5

TABLE I

	Purification Step	Volume in Liter	Total Protein, mg	Total Activity, CEL I units	Specific Activity, units/mg	Protein, Fold-Purification
10	Buffered Juice	79.34	19,399	1.9×10^7	9.7×10^2	
	25% $(\text{NH}_4)_2\text{SO}_4$ Supernatant	70.56	17,005	1.6×10^7	9.2×10^2	1
15	80% $(\text{NH}_4)_2\text{SO}_4$ pellet	8	2,072	9.0×10^6	4.4×10^3	4.5
	ConA-Sephrose 4B	2.5	6.75	3.6×10^6	5.4×10^5	553.8
20	DEAE-Sephacel	0.12	2.69	2.4×10^6	8.8×10^5	907.6
	Phospho-cellulose P-11	0.48	0.408	1.5×10^6	3.8×10^6	3,854
25	Phenol Sepharose CL-4B	0.34	0.054	5.6×10^5	1.0×10^7	10,676
	Mono Q	0.03	0.03	3.6×10^5	1.2×10^7	12,316
	Superdex 75	0.0005	0.005	3.1×10^5	3.1×10^7	33,000

30

EXAMPLE 2

Isoelectric point of CEL I and CEL II

A sample of CEL I, containing a small amount of CEL II, was loaded onto an isoelectric focusing gel (pH 3-10, from Novex). After the gel was stained, the pI of the CEL I and CEL II were obtained by comparison with the standards (Bio-Rad). The pI of the CEL I band was between 6.0 and 6.5, and the pI of the CEL II band was between 6.5 and 6.8 (data not shown). After minimizing the N-linked oligosaccharides by Endo H_f, the 43 KDa major celery nuclease band shifted to the 29 KDa

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5 position (Fig. 1B & C, lanes 4) and the 39 KDa minor
celery nuclease band shifted to the 37 KDa position
(Fig. 1C, lane 4). If CEL II were a degradation product
of CEL I, after endo H_f treatment, its polypeptide length
should be equal or less than 29 KDa.

10

EXAMPLE 3

Effects of Reducing Agents on CEL I

When 1 % -mercaptoethanol was used in the sample
buffer for SDS-PAGE analysis of the CEL I band, CEL I
15 was shifted upward (Fig 1D, lane 2) but intact. DTT was
also tested and similar results were obtained (data not
shown). The simplest interpretation is that the CEL I
polypeptide does not contain any breakage in the
backbone. Instead, disulfide bonds were broken that
20 resulted in the enzyme becoming more extended in the
reduced state, and hence slower in electrophoretic
mobility.

EXAMPLE 4

Renaturation of homogeneous CEL I and CEL II

25 Individual celery nuclease bands were excised from
the 10% SDS-PAGE and eluted as described in above. These
bands included the 43 KDa band, the 39 KDa band, and
their corresponding bands after the Endo H_f digestion.
The eluted enzyme fractions were concentrated and
30 renatured. Plasmid nicking assays were carried out to
show that the renatured samples were all active
nucleases. See Figure 9. The renatured CEL I before or
after Endo H_f digestion and CEL II after Endo H_f digestion
were able to incise DNA at a mismatch substrate. In this
35 experiment, the mismatch incised is a G residue
insertion. This experiment is necessarily qualitative
because of the uncertainties in the recovery of proteins
and activity in the gel elution and renaturation steps.

- 55 -

5 However, the data strengthens the conclusion that CEL I and CEL II are homogeneous and each able to incise at a DNA mismatch, and that most of the carbohydrates on CEL I and CEL II are not essential for activity.

10

EXAMPLE 5***The cloning of CEL I cDNA***

 The amino acid sequence of the N-terminal and three other internal proteolytic peptides of CEL I, identified by Edman degradation performed by the Protein/DNA
15 Technology Center of the Rockefeller University, are shown in Fig. 2 in bold letters. The 72 amino acids identified represent about 28% of the CEL I polypeptide and were completely accounted for in the cDNA sequence. CEL I without the leader sequence is a protein of 274
20 amino acid residues, with a calculated molecular weight of 31,440.2. Compared with the apparent molecular weight of 43 KDa determined in SDS PAGE, CEL I is 27 percent carbohydrate by weight.

 Alignment of the CEL I cDNA amino acid sequence
25 with homologs in Genbank by the PSI-Blast program at NCBI (20) revealed that CEL I has relative low identity to the *Aspergillus* S1 nuclease (accession P24021, 27% of 273 amino acids) and P1 nuclease (accession P24289, 30% of 277 amino acids) See Figure 10. However, among all
30 the homologs of CEL I in plants, three stand out to be of very high degree of identity. Namely, ZEN1 (accession AB003131, 80% identity of 269 amino acids), DSA6 (accession AF082031, 73% identity of 271 amino acids), BFN1 (accession U90264, 72% identity of 274 amino
35 acids). We propose that these three proteins are probably orthologs of CEL I because all other homologs are at a range of 45% identity or lower. Moreover, when one superimposes the sequences of CEL I and these three

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5 orthologs on the secondary structure of P1 nuclease,
most of the sequence differences among these four
putative orthologs are in the flexible loop regions that
connect consecutive helices (Fig. 10) and in the
flexible COOH terminal region. Thus it is very likely
10 that these orthologs share the enzymatic properties of
CEL I and may not share the catalytic properties and
substrate specificity of the S1 nuclease.

15 **EXAMPLE 6**

Mg⁺⁺ and pH dependence of CEL I

A gel-image of the automated DNA sequencer analysis
of the CEL I incision at the mismatch of a T@G base
substitution is shown in Fig. 3. Lanes 1-4 are mock
20 reactions without CEL I. The full length 235 nt PCR
product is seen on top of the image, and imperfect PCR
products are seen as the bands dispersed below. In lane
5, in the presence of CEL I, Mg⁺⁺ and pH 7.5, the blue
incision band of 156 nt and the green incision band of
25 80 nt are observed as indicated. In the absence of Mg⁺⁺
or in pH 5.5 (Lanes 6-8), mismatch-specific incisions
are not significant. This experiment also illustrates
how the imperfect PCR byproducts seen in lanes 1-4 are
eliminated by CEL I in lanes 5-8, especially under the
30 conditions of lane 5.

EXAMPLE 7

The RF-I nicking activity of CEL I and MBN

Supercoiled plasmid replicative form I (RF-I) DNA
35 exhibit local regions of instability in the double-helix
that can be attacked by nucleases. Upon the first nick,
the superhelical stress is relieved, and the DNA is no
longer a substrate for most single-strand nucleases. The

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5 RF-I nicking activities of MBN and CEL I at pH 5.5
versus pH 7.5 are shown in Fig. 4. Panel A and B compare
the nicking of RF-I by MBN-A at the two pH's in the
presence and absence of Mg^{++} . In panel A, under condition
of initial kinetics, the inhibition of MBN by 3 mM Mg^{++}
10 is about 90%. About 70% of the RF-I is nicked by 7 pg of
MBN-A in 30 min at pH 5.5. In panel B, 7 ng of MBN-A can
only nick about 20% of the RF-I in 30 min at pH 7.5.
Similar result is obtained for MBN-B in panels C and D.
Similar comparison of CEL I RF-I nicking activity is
15 shown in panel E for pH 5.5, and panel F for pH 7.5. The
data shows that CEL I is about twice as active in RF-I
nicking in the presence of Mg^{++} than in the absence of
 Mg^{++} . Comparing the 5 pg data points, CEL I is twice more
active at pH 7.5 than at pH 5.5.

20

EXAMPLE 8

The single-strand DNase activity of CEL I and MBN

The digestion of denatured purified calf thymus DNA
by MBN and CEL I is shown in Fig. 5. For ease of
25 comparison, different amounts of MBN and CEL I were used
so that the assays are in a similar range of total
activity. The amounts of enzyme used for MBN-A, MBN-B,
and CEL I were 0.7 ng, 1.9 ng, and 16 ng, respectively.
The lack of activity by MBN at pH 7.5 is obvious in
30 panels A and B. The Mg^{++} inhibition of MBN is also
observed for the activity on single-stranded DNA. In
contrast, CEL I is more active in the presence of Mg^{++}
than in the absence. Importantly, comparing the initial
kinetics in panels A and C for the highest activity
35 condition for each enzyme, MBN-A in the absence of Mg^{++}
at pH 5.5 appears to be about 32 times higher in single-
strand nuclease specific activity than for CEL I in the
presence of Mg^{++} at pH 5.5 (1.42×10^6 g DNA

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5 solubilized/min/mg protein versus 4.46×10^4 g DNA
solubilized/min/mg protein).

EXAMPLE 9

The mismatch endonuclease activity of CEL I and MBN

10 The nicking of DNA duplexes containing mismatches
by MBN and CEL I is shown in Fig. 6. The mismatch with a
four base loop is nicked by CEL I and both preparations
of MBN at pH 7.5 (A, B, C). Note the higher amounts of
MBN needed in this reaction. However, even at 1000
15 times more enzyme than CEL I, MBN is unable to
specifically nick at base-substitutions at a single base
mismatch (D, E, G, and H). When the same amount of MBN
protein is incubated with DNA substrates at pH 5.5 as at
pH 7.5 the substrate is almost completely digested (data
20 not shown). When a lesser, more appropriate amount of
MBN is incubated with the DNA substrate at pH 5.5, no
mismatch-specific nicking is seen (data not shown). CEL
I nicks at the base-substitution mismatch (panel F) and
at the extrahelical nucleotide (panel I). In panel F,
25 the blue peak at position 183 nt corresponds to the nick
at the 3' side of the mismatch on the 6-FAM-labeled
strand of the heteroduplex, and the green peak at
position 142 nt corresponds to the nick at the 3' side
of the mismatch on the TET-labeled strand. Some of the
30 other blue peaks are non-specific cutting by CEL I; it
is important to note that if one incubates the reaction
for a longer time, or with more CEL I enzyme, most of
these non-mismatch specific peaks will be removed while
the mismatch-specific peaks will remain (Fig. 3). The
35 reason is that these background bands are often non-
specific heteroduplexes of PCR products in which the two
DNA strands do not basepair properly. These duplexes are
nicked by CEL I at non-specific positions, and their

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5 signal becomes diffused. In panel I, the green peak at
252 nt corresponds to the nick at the 3' side of the
extrahelical G on the TET-labeled strand of the PCR
product. A blue peak corresponding to the nick at the
extrahelical C on the 6-FAM-labeled strand is expected
10 at position 151 nt, but is not seen. CEL I may have
nicked the 6-FAM-labeled strand near its 5'-end removing
the dye, making it unable to score the blue peak in the
assay. Alternatively, the insert C substrate may have
been out-competed by the insert G substrate.

15

EXAMPLE 10

The RNase activity of CEL I and MBN

A property common to S1 and CEL I is the ability to
digest both RNA and DNA, a feature referred to as "sugar
20 non-specific" or "bifunctional" in literature. We have
compared the specific activities of MBN and CEL I on RNA
using conditions comparable to their DNase activities.
The specific questions addressed here are whether the
RNase activity is pH-dependent, and whether the specific
25 activities of the RNase and DNase are similar for each
enzyme. Our assay measures the digestion of RNA to
soluble nucleotides and short RNA fragments. The
specific activity of the RNase activity of MBN-A (Fig.
7A) is comparable to its single-strand DNase activity
30 (Fig. 5A). The specific activity of CEL I is 50 times
less than MBN-A on *Torula* Yeast RNA (Fig. 7A) at pH 5.5.
This value is consistent with our finding that CEL I is
about 32 times lower in specific activity than MBN-A
using denatured calf-thymus DNA as substrate. CEL I as
35 an RNase is slightly more active at pH 7.5 than at pH
5.5. This is opposite to the observation for the single-
strand DNase activity of CEL I, but the differences are
small. Thus MBN at pH 5.5, and CEL I at pH 5.5 and pH

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5 7.5, showed no preference for RNA versus DNA. MBN-A
digested RNA at pH 7.5 with the same specific activity
as at pH 5.5 (Fig. 7). This is in striking contrast to
MBN-A's little to no ability to digest single-stranded
DNA at pH 7.5 (Fig. 5A). Similar results were found for
10 the RNase activity of MBN-B (data not shown).

DISCUSSION

The purification of glycoproteins

15 We previously described a purification protocol
that produced highly enriched CEL I, however the enzyme
never appeared as a single band on a SDS PAGE gel (8).
To identify the source of contamination, we repeated the
purification with *Arabidopsis* callus, and observed the
20 same problem of aggregation. We made mouse antibodies to
the purest fraction and used the antiserum to identified
clones of two different genes from an *Arabidopsis* cDNA
expression library (21) (accession # AC001645, genes
PID:g2062157 and PID:g2062159) (unpublished data). These
25 clones were found to be highly homologous to two
jasmonate inducible proteins of *Brassica napus* that are
known to function as ConA-like lectins (accession #
CAA72271, 62% identity in 475 amino acids) (22). Such
lectins are coded for by over 30 genes in *Arabidopsis*
30 and can be a problem when the glycoprotein to be
purified is less abundant than the lectins. The presence
of mannose in the buffers in the present protocol has
overcome this obstacle and has provided a homogeneous
preparation of CEL I.

35

Alignment of CEL I Amino Acid Sequence

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5 In an alignment of CEL I amino acid sequence with
all the S1 homologs in Genbank, (Fig.10), the
universally conserved residues are the N-terminal
tryptophan residue, five histidine residues, and three
aspartate residues, located in different regions of the
10 polypeptide (Fig. 2). These nine residues are brought
together to bind the three Zn⁺⁺ atoms, as revealed by the
X-ray crystallography structure of the P1 nuclease (23-
24). The conservation of the catalytic active site
suggests that these nucleases share the same mechanism
15 for the cleavage of the phosphodiester bonds,
necessitating the conservation of the enzyme structure
to form the catalytic domain. The differences in
substrate preference may lie in the mechanism of
substrate recognition, separate from catalysis, such
20 that S1 family nucleases are specific for single-
stranded nucleic acids whereas CEL I shows high
specificity for mismatch heteroduplexes. The sequences
that enable the recognition of different substrates may
reside in amino acid sequences that are less conserved.
25 To better define the catalytic differences of CEL I and
S1 type nucleases, we performed a careful contrast of
CEL I with MBN, the best characterized ortholog of S1
nuclease in plant.

30 *The pH dependence of CEL I and Mung Bean Nuclease*

 In the RF-I of plasmid pUC19, supercoiling induces
regions of single-strandedness that can become a
substrate for nucleases. Moreover, regions such as the
origin of replication are known to form stem-loop
35 structures. It has also been shown that there are
destabilized sequences in supercoiled plasmids (25). The
data in Fig. 4 demonstrated that MBN nicks RF-I more

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5 quickly at pH 5.5 than at pH 7.5 by more than 1000 fold,
yet CEL I is more active at pH 7.5 than at pH 5.5.

The >1000 fold higher activity of MBN at acidic pH
on RF-I cutting may be a function of the catalytic
mechanism of the enzyme. Another factor that contributes
10 to faster rate of RF-I nicking at acidic pH may be the
partial unwinding of a plasmid at acidic pH, thereby
producing a greater propensity for single-strandedness.
In the case of CEL I being active on plasmid RF-I at
neutral pH, one may speculate that a partial unwinding
15 of the RF-I occurs upon the binding of CEL I.
Alternatively, CEL I may not be recognizing single-
strandedness in the plasmid. The reason is that in spite
of CEL I being more active in the digestion of single-
stranded DNA at pH 5.5 than at pH 7.5 (Fig. 5), CEL I is
20 less active in RF-I nicking at pH 5.5 than at pH 7.5
(Fig. 4).

When CEL I uses denatured DNA as a substrate, the
specific activity of CEL I is 20 times less than MBN-A
(Fig. 5C) at acidic pH and only slightly improved at pH
25 7.5 in the presence of Mg^{++} . In RF-I nicking, which
reflects the recognition of destabilized helices, CEL I
specific activity is only 2 times less than MBN-A at pH
5.5, but CEL I is 1000 times more active at pH 7.5 (Fig.
4). Moreover, CEL I nicks a mismatch heteroduplex
30 containing four extrahelical bases at 700 times higher
specific activity than MBN-A (Fig. 6A, B, C). Lastly,
only CEL I can nick DNA at base-substitutions.
Therefore, it is evident that CEL I is not primarily a
single-strand DNase. Moreover, single-strandedness per
35 se is not what CEL I recognizes in a mismatch substrate.

The role of Mg^{++} in the activity CEL I and the MBN

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5 The initial rate of RF-I nicking by MBN at pH 5.5
is inhibited by Mg^{++} by about 10 to 20 fold. In contrast,
CEL I is stimulated by Mg^{++} under all assay conditions.
The CEL I nicking of RF-I significantly increases in the
presence of Mg^{++} at both pH's. By the RF-I nicking assay
10 itself, it is not possible to distinguish whether the
effect of the Mg^{++} is on the plasmid DNA structure or on
the enzyme. With single-stranded DNA as substrate, the
effect of Mg^{++} on the enzymes was lower perhaps because
the effects of Mg^{++} on substrate superhelicity is not
15 involved. With the mutation detection assay, it is clear
that Mg^{++} is required for optimal CEL I incision at
mismatches in double-stranded DNA (Fig. 3). If CEL I and
MBN should use the same catalytic mechanism for
phosphodiester bond cleavage, their differences may lie
20 in how the substrates are recognized. The role of Mg^{++}
may lie in a structural role for substrate recognition
and not in DNA hydrolysis (26). Lastly, both MBN and CEL
I are observed to be RNases. Surprisingly, MBN is
primarily an RNase at neutral pH with the RNase activity
25 at least one thousand times greater than the DNase
activity.

Thus it is clear that MBN and CEL I represent two
different enzyme families within the S1 superfamily of
structurally related nucleases. The high resolution X-
30 ray structure of the P1 nuclease showed that a double
stranded helix cannot fit into the P1 DNA binding grove
(21-22).

In summary, it appears that CEL I exemplifies a
unique family of mismatch recognizing nucleases.
35 Additionally, based on marked sequence similarity, it
appears that CEL I ortholog sequences may be also used
to advantage in the assay methods for mutation
detection as described herein for CEL I.

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5

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- 50 While certain preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made to the invention without departing from the scope and spirit thereof as set forth in the following claims.

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5 **WHAT IS CLAIMED IS:**

1. An isolated nucleic acid molecule having the sequence of SEQ ID NO:1, said nucleic acid molecule encoding an endonuclease protein from celery about 309 amino acids in length, said encoded protein comprising a plurality of α helix domains and a flexible carboxy terminal region.

2. The nucleic acid molecule of claim 1, which is DNA.

3. The DNA molecule of claim 2, which is a cDNA comprising a sequence approximately 1135 base pairs in length that encodes said endonuclease protein.

4. The DNA molecule of claim 2, which is a gene comprising introns and exons, the exons of said gene specifically hybridizing with the nucleic acid of SEQ ID NO:1, and said exons encoding said endonuclease protein.

5. An isolated RNA molecule transcribed from the nucleic acid of claim 1.

6. A polynucleotide which comprises:

a) a sequence encoding a protein or polypeptide as defined in claim 1;

b) a sequence encoding the complementary sequence of a);

c) a sequence of nucleotides shown in Figure 2;

d) a sequence capable of selectively hybridizing to a sequence in either a) or c); and

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5 e) a fragment of any of the sequences in a),
b) or c).

7. The nucleic acid molecule of claim 6,
wherein said nucleic acid molecule encodes an
10 endonuclease protein comprising an amino acid sequence
selected from the group consisting of an amino acid
sequence having SEQ ID NO:2 and amino acid sequences
encoded by natural allelic variants of said nucleic acid
molecule.

15 8. The nucleic acid molecule of claim 7,
which comprises SEQ ID NO:1.

9. An oligonucleotide between about 10 and
20 about 200 nucleotides in length, which specifically
hybridizes with SEQ ID NO:1.

10. A nucleic acid molecule encoding an
endonuclease having the sequence of SEQ ID NO: 2.

25 11. An antibody immunologically specific for
the isolated protein encoded by the nucleic acid
molecule of claim 10.

30 12. An antibody as claimed in claim 11, said
antibody being monoclonal.

13. An antibody as claimed in claim 11, said
antibody being polyclonal.

35 14. A plasmid comprising SEQ ID NO: 1.

15. A vector comprising SEQ ID NO: 1.

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5 16. A retroviral vector comprising SEQ ID NO:
1.

 17. A host cell comprising a nucleic acid
molecule having the sequence of SEQ ID NO:1.

10

 18. A host cell as claimed in claim 17,
wherein said host cell is selected from the group
consisting of bacterial, fungal, mammalian, insect and
plant cells.

15

 19. A host animal comprising SEQ ID NO: 1.

 20. A method for screening a test compound
for CEL I modulating activity, comprising:

20 a) providing a host cell expressing a CEL I
encoding nucleic acid;

 b) contacting said host cell with a compound
suspected of modulating CEL I activity; and

 c) determining the CEL I modulating activity
25 as assessed by an alteration in the endonuclease
activity of CEL I.

 21. A method for determining a mutation in a
target sequence of single stranded polynucleotide with
30 reference to a non-mutated sequence of a polynucleotide
that is hybridizable with the polynucleotide including
said target sequence, wherein said sequences are
amplified, labeled with a detectable marker, hybridized
to one another, exposed to endonuclease and analyzed for
35 the presence of said mutation, the improvement
comprising a nucleic acid which encodes an amino acid
sequence greater than 60% identical to SEQ ID NO: 2, and
which encodes a mismatch endonuclease for production of

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5 large quantities of said endonuclease, the activity of said endonuclease comprising:

- a) detection of all mismatches between said hybridized sequences;
- b) recognition of sequence differences in
10 polynucleotide strands between about 100bp and about 3kb in length; and
- c) recognition of said mutation in a target polynucleotide sequence without substantial adverse effect caused by flanking polynucleotide sequences.

15

22. The method as claimed in claim 21, wherein said nucleic acid sequence, which encodes an amino acid sequence having greater than 60% identity to
20 SEQ ID NO: 2, is selected from the group of sequences consisting of nucleic acid sequences encoding BFN1, ZEN1 and DSA6.

23. A method for determining a mutation in a
25 target sequence of single stranded polynucleotide with reference to a non-mutated sequence of a polynucleotide that is hybridizable with the polynucleotide including said target sequence, wherein said sequences are amplified, labeled with a detectable marker, hybridized
30 to one another, exposed to endonuclease and analyzed for the presence of said mutation, the improvement comprising a nucleic acid having a sequence of SEQ ID NO: 1 which encodes a mismatch endonuclease for production of large quantities of said endonuclease, the
35 activity of said endonuclease comprising:

- a) detection of all mismatches between said hybridized sequences;

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5 b) recognition of sequence differences in
polynucleotide strands between about 100bp and about 3kb
in length; and

 c) recognition of said mutation in a target
polynucleotide sequence without substantial adverse
10 effect caused by flanking polynucleotide sequences.

24. The method as claimed in claim 23 wherein
said endonuclease is derived from celery.

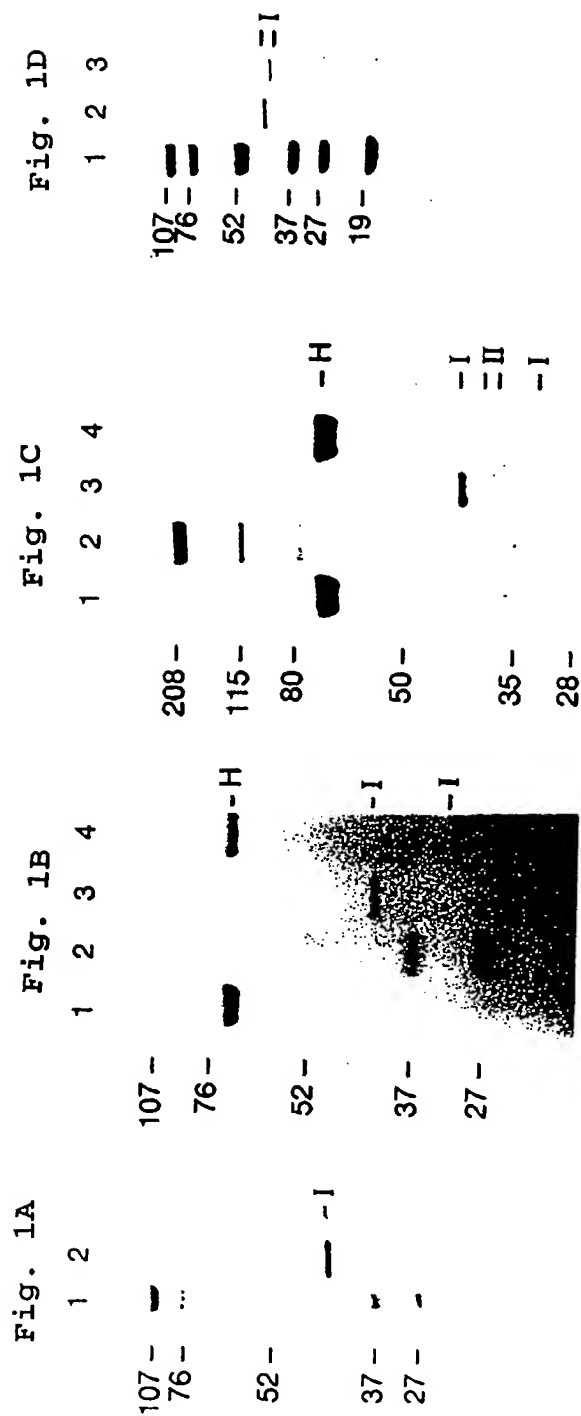
15 25. The method as claimed in claim 23 wherein
said polynucleotide is DNA.

26. The method as claimed in claim 23 wherein
said polynucleotide is cDNA.

20 27. A method as claimed in claim 28, wherein
said mutation is indicative of a genetic disorder.

28. A method as claimed in claim 28, wherein
25 said mutation indicative of a predisposition to cancer.

29. An isozyme of CEL I having endonuclease
activity, said isozyme having a molecular weight of 39
kd and being isolated from celery.



1 GACAAGCGCCATCTATGAGTTTCATCATGCCCTATATATAAACACATGAACCTGTCATTGT 60
61 TCATTTATGCATTATTGTTGTATTAGCTGAAAAATTTCTGGCAAATGACGCGATTATATT 120
M T R L Y S -
121 CTGTGTTCTTTCTTTTGTGGCTCTTGTAGTTGAACCGGGTGTAGAGCCTGGAGCAAAG 180
V F F L L L A L V V E P G V R A W S K E -
↑+
181 AAGGCCATGTCATGACATGTCAAATTGCGCAGGATCTGTTGGAGCCAGAAGCAGCACATG 240
G H V M T C Q I A Q D L L E P E A A H A -
+
241 CTGTAAAGATGCTGTTACCGGACTATGCTAATGGCAACTTATCGTCGCTGTGTGTGGC 300
V K M L L P D Y A N G N L S S L C V W P -
301 CTGATCAAATTCGACACTGGTACAAGTACAGGTGGACTAGCTCTCTCCATTTTCATCGATA 360
D Q I R H W Y K Y R W T S S L H F I D T -
+ +
361 CACCTGATCAAGCCTGTTTCATTGATTACCAGAGAGACTGTCATGATCCACATGGAGGGA 420
P D Q A C S F D Y Q R D C H D P H G G K -

421 AGGACATGTGTGTTGCTGGAGCCATTCAAATTTACATCTCAGCTTGGACATTTCCGCC 480
D M C V A G A I Q N F T S Q L G H F R H -

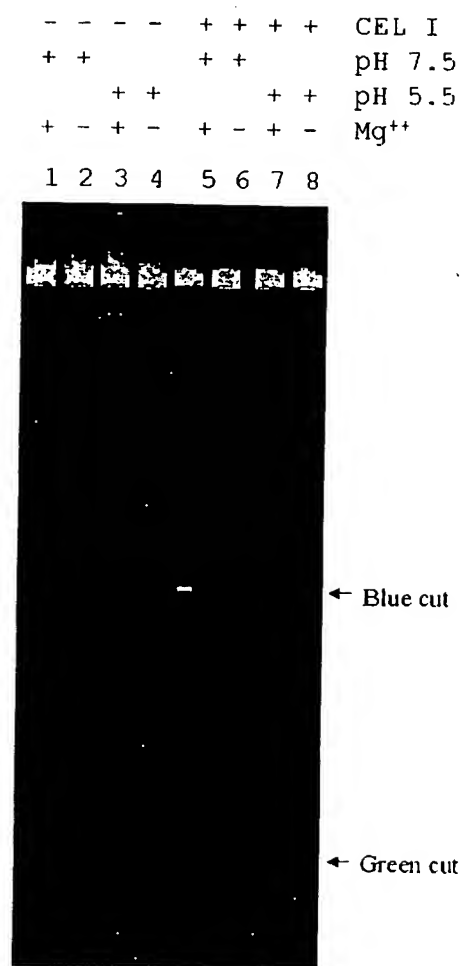
481 ATGGAACATCTGATCGTCGATATAATATGACAGAGGCTTTGTTATTTTTATCCCACTTCA 540
G T S D R R Y N M T E A L L F L S H F M -
+
541 TGGGAGATATTCATCAGCCTATGCATGTTGGATTTACAAGTGATATGGGAGGAAACAGTA 600
G D I H Q P M H V G F T S D M G G N S I -
+ +
601 TAGATTTGCGCTGGTTTTCGCCACAAATCCAACCTGCACCATGTTTGGGATAGAGAGATTA 660
D L R W F R H K S N L H H V W D R E I I -
+ +
661 TTCTTACAGCTGCAGCAGATTACCATGGTAAGGATATGCACTCTCTCCTACAAGACATAC 720
L T A A A D Y H G K D M H S L L Q D I Q -
721 AGAGGAACCTTTACAGAGGGTAGTTGGTTGCAAGATGTTGAATCCTGGAAGGAATGTGATG 780
R N F T E G S W L Q D V E S W K E C D D -
781 ATATCTCTACTTGCGCCAATAAGTATGCTAAGGAGAGTATAAACTAGCCTGTAAGTGGG 840
I S T C A N K Y A K E S I K L A C N W G -

841 GTTACAAAGATGTTGAATCTGGCGAAACTCTGTGAGATAAATACTTCAACACAAGAATGC 900
Y K D V E S G E T L S D K Y F N T R M P -
901 CAATTGTCATGAAACGGATAGCTCAGGGTGAATCCGTTTATCCATGATTTTGAACCGAG 960
I V M K R I A Q G G I R L S M I L N R V -
961 TTCTTGAAGCTCCGCAGATCATTCTTTGGCATGAATTTAGATACTGATATTCGCATTTTC 1020
L G S S A D H S L A *
1021 TCATGACACCCTTCTCTTATGCAATTTGCAGATCAGCTGTGATTCACTAATTGAA 1075

Figure 2

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Figure 3



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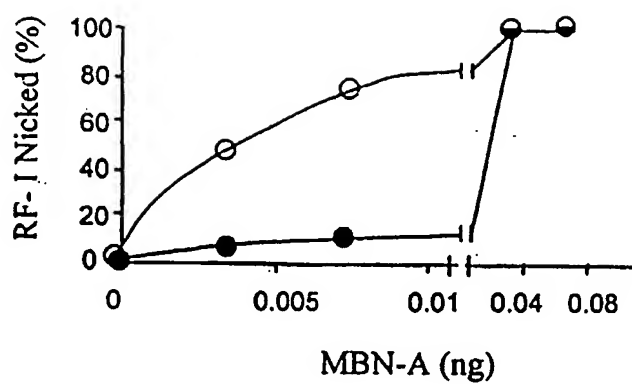


Fig. 4A

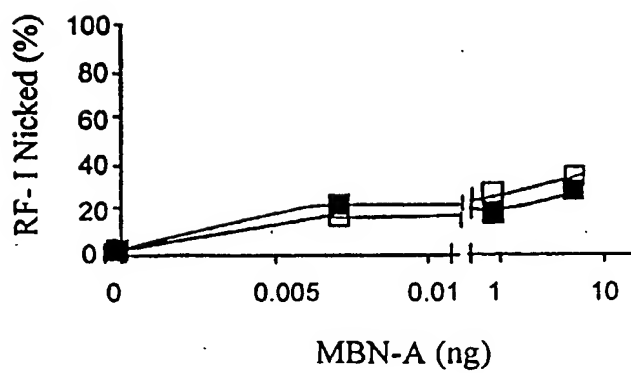


Fig. 4B

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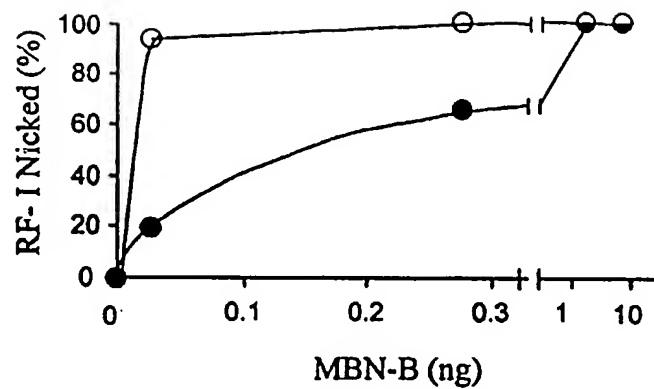


Fig. 4C

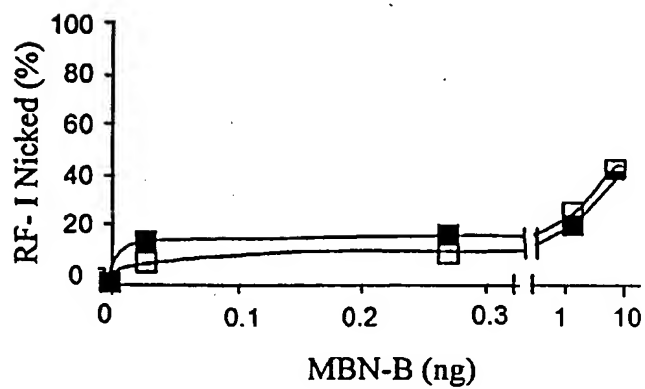


Fig. 4D

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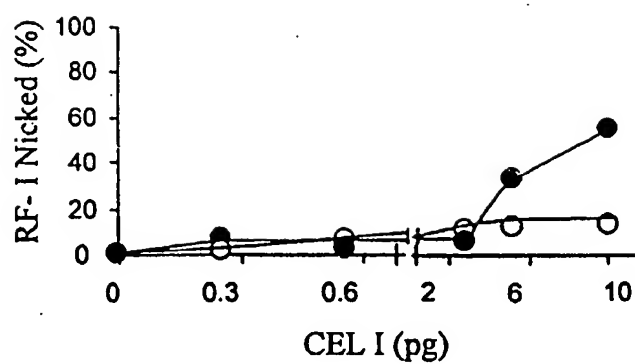


Fig. 4E

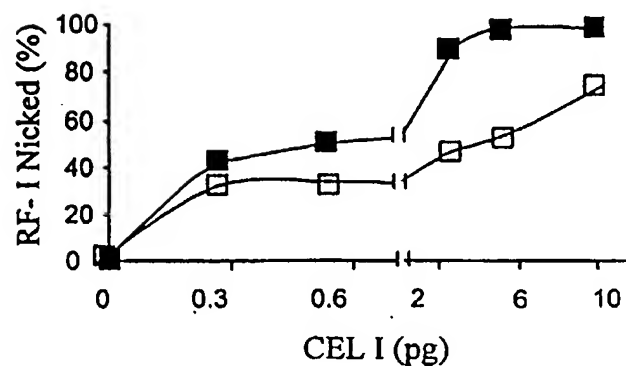


Fig. 4F

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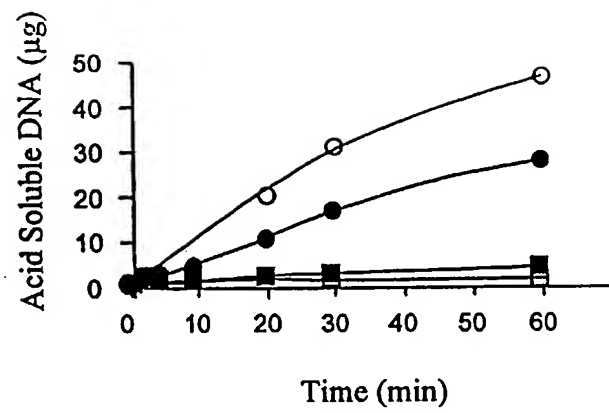


Fig. 5A

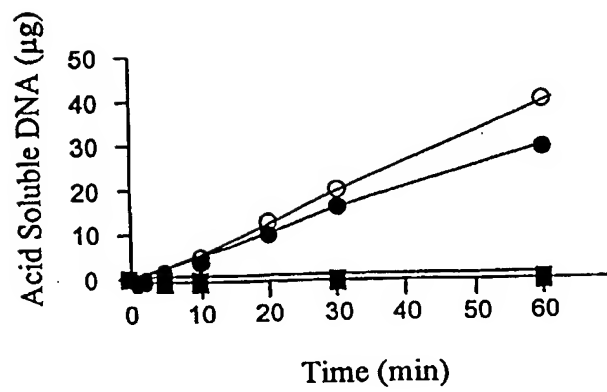


Fig. 5B

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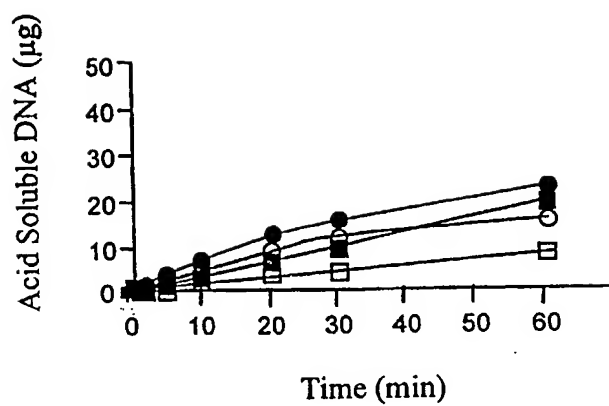


Fig. 5C

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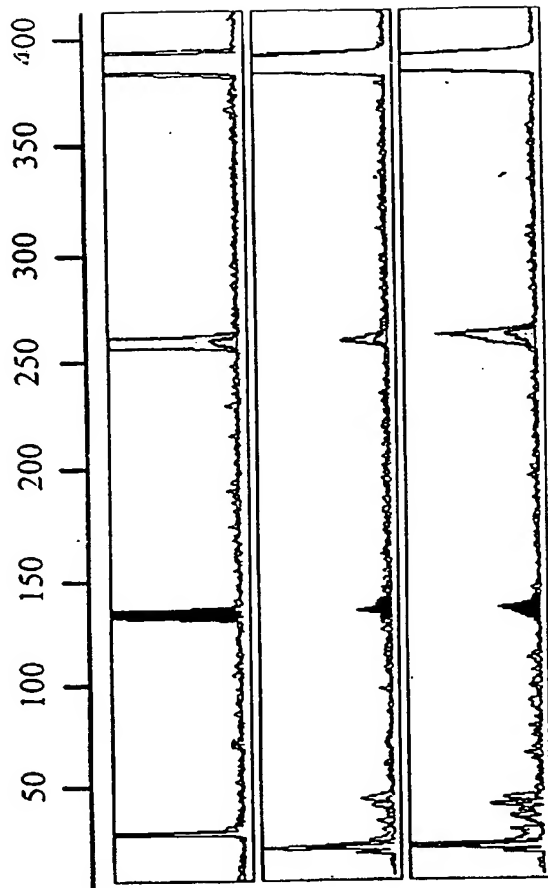


Fig. 6A

Fig. 6B

Fig. 6C

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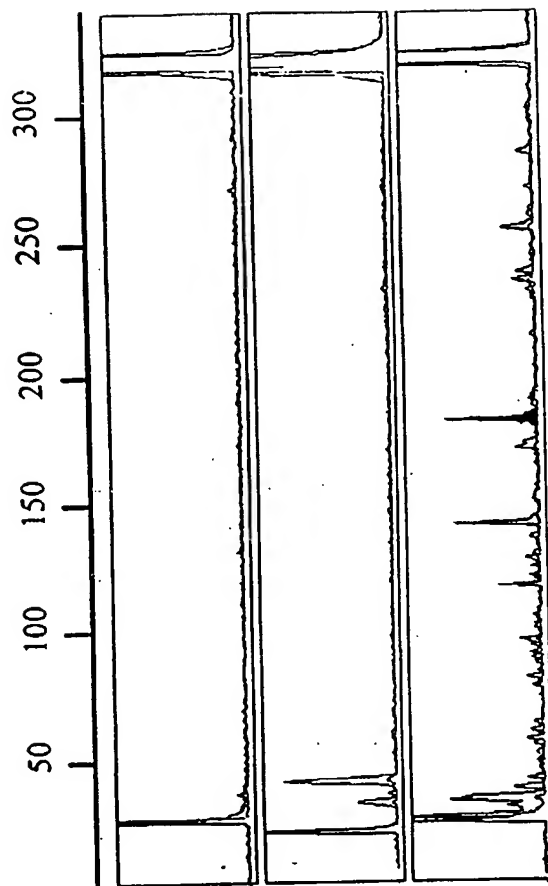


Fig. 6D

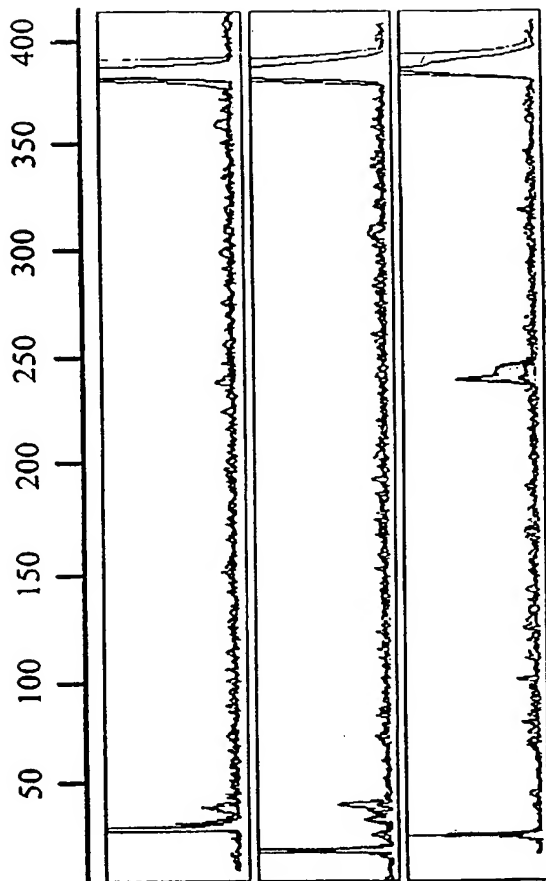
Fig. 6E

Fig. 6F

Fig. 6G

Fig. 6H

Fig. 6I



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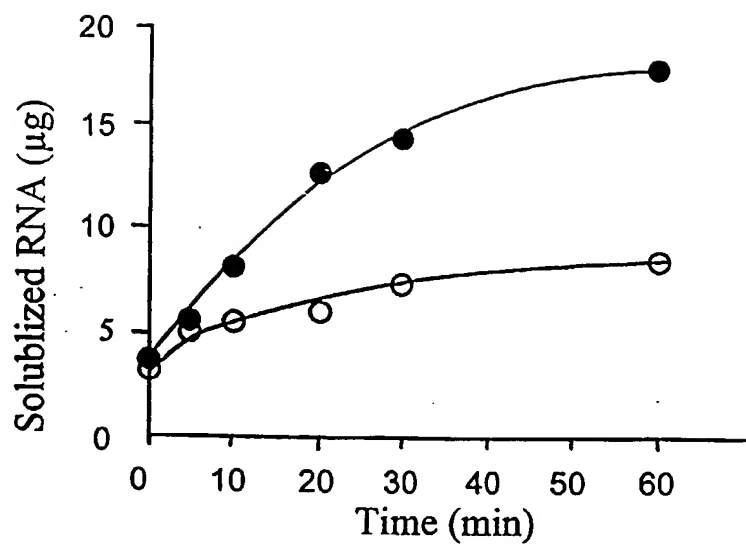


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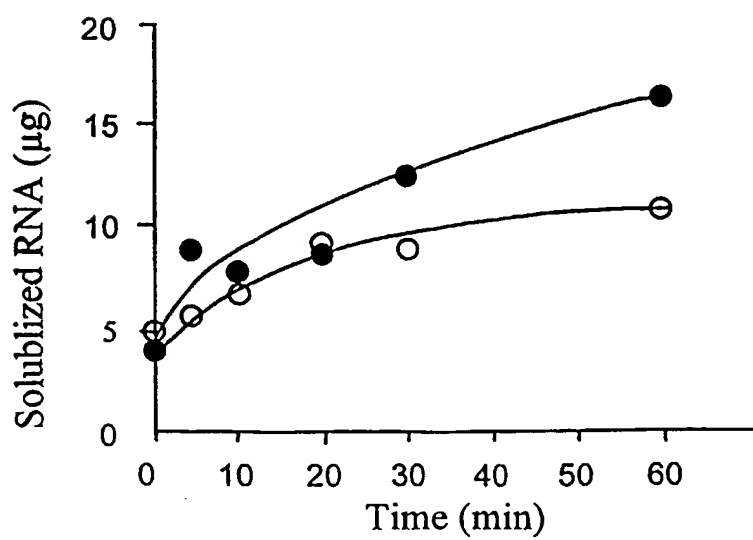


Fig. 7B

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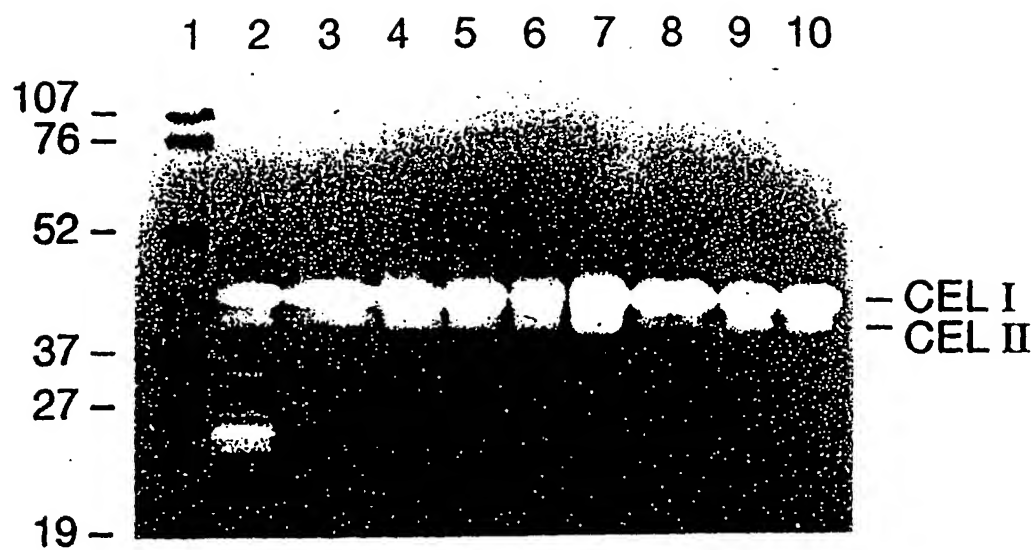


Figure 8

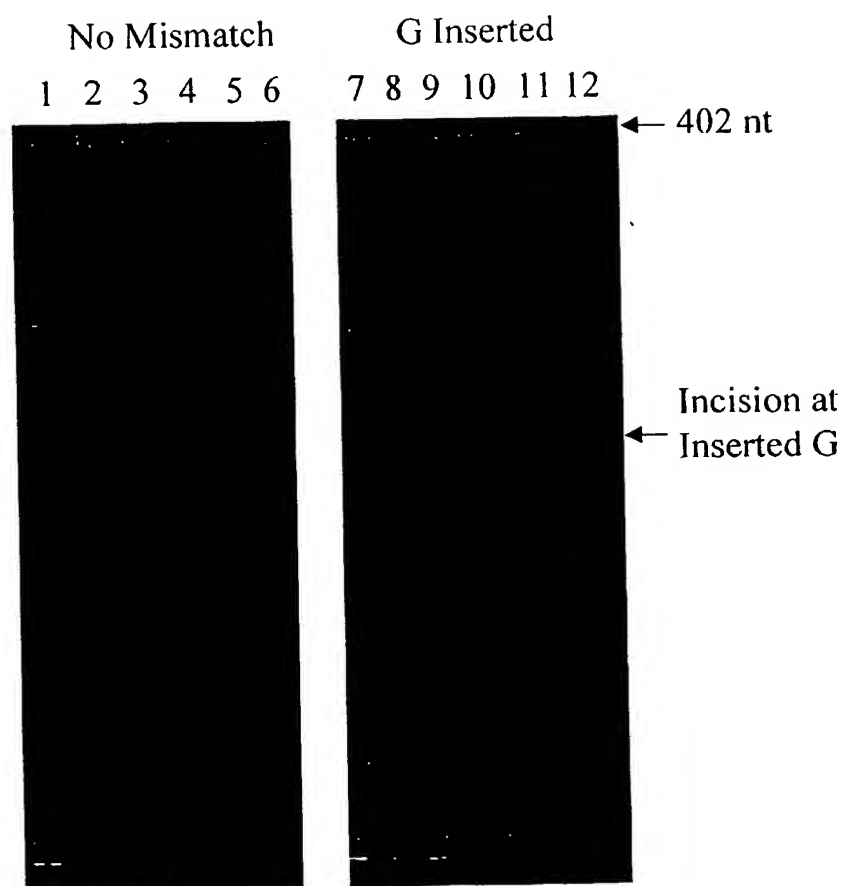


Fig. 9A

Fig. 9B

Fig. 10

Fig. 10

SEQUENCE LISTING

<110> Fox Chase Cancer Center

<120> Nucleic Acid Encoding a Mismatch
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<130> FCCC 95-03 PCT

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Thr Thr Asn Leu His His Ile Trp Asp Thr Asn Met Pro Glu Glu Ala
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Pro Asp Asp Ala Cys Ser Phe Asp Tyr Ser Arg Asp Cys His Asp Pro
      65      70      75      80
Lys Gly Ala Glu Asp Met Cys Val Ala Gly Ala Val His Asn Tyr Thr
      85      90      95
Thr Gln Leu Met His Tyr Arg Asp Gly Thr Ser Asp Arg Arg Tyr Asn
      100      105      110
Leu Thr Glu Ser Leu Leu Phe Ser Leu His Phe Met Gly Asp Ile His
      115      120      125
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/05502

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; C07H 21/04

US CL : 435/6; 536/22.1+

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/22.1+

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

435/91.2, 195, 196

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, BIOSIS, CAPLUS, EMBASE

search terms: endonuclease, mutation, CEL I, plant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,869,245 A (YEUNG) 09 February 1999(09.02.99), see entire document.	20-29 ----- 1-19
X ---- Y	OLEYKOWSKI.C.A. et al. Mutation detection using a novel plant endonuclease. Nucleic Acids Res. August 1998, Vol. 20, No. 20, pages 4597-4602, see entire document.	20-29 ----- 1-19
X,P	KULINSKI. J. et al. CEL I Enzymatic Mutation Detection Assay. BioTechniques. July 2000, Vol. 29, No. 1, pages 44, 46 and 48, see entire document.	1-29

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

11 JUNE 2001

Date of mailing of the international search report

12 JUL 2001

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

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